

# **Dopaminergic signalling in *Drosophila melanogaster* – Insights into the control of metabolism and behaviour**

**Dopaminvermittelte Signalwege in *Drosophila melanogaster* –  
Einblicke in die Steuerung von Metabolismus und Verhalten**

**Dissertation**

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## ABBREVIATIONS

abdg	abdominal ganglion
AC	adenylate cyclase
ant	anterior
ATP	adenosine triphosphate
a. u.	arbitrary units
AVLP	anterior ventrolateral protocerebrum
bPAC	bacterial photoactivated adenylate cyclase
°C	degrees celcius
cAMP	cyclic adenosine 3', 5' monophosphate
CNS	central nervous system
CO <sub>2</sub>	carbon dioxide
Ca <sup>2+</sup>	calcium ions
ChR2	Channelrhodopsin-2
d	days
DA	dopamine
DAT	dopamine transporter
dFSB	dorsal fan-shaped body
dor	dorsal
D2R	dopamine 2-like receptor
dsRNA	double-stranded RNA
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
GIT	gastrointestinal tract
G-protein	GTP binding protein

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GPCR	G protein coupled receptor
h	hour(s)
H <sup>+</sup>	hydrogen ion
HR	hazard ratio
H-B	Hofbauer-Buchner tract
IIS/TOR	insulin/insulin-like growth factor signalling/target of rapamycin
IR	immunoreactivity
L	lamina
L-dopa	L-3',4'-dihydroxyphenylalanin
LN <sub>v</sub> s	lateral ventral neurons
LO	lobula
lx	lux
ME	medulla
med	medial
min	minutes
max.	maximum
meso	mesothorax
meta	metathorax
mg	milligram
Na <sup>+</sup>	sodium ion
ns	not significant
OL	optic lobe
O <sub>2</sub>	oxygen
PB	protocerebral bridge
PD	Parkinson's disease

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PDF	pigment dispersing factor
PER	proboscis extension reflex
PNS	peripheral nervous system
pro	prothorax
px	pixel
RNAi	RNA interference
sd	standard deviation
TAG	triacylglyceride
TH	tyrosine hydroxylase
SEM	standard error of the mean
SP	survival phase
TAG	triacylglyceride
μl	microlitre
ven	ventral
VMAT-2	vesicular monoamine transporter 2
VNC	ventral nerve cord
zt	zeitgeber time

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## SUMMARY

The biogenic amine dopamine (DA) is a central messenger implicated in various physiological tasks from invertebrates to men. Its major neurotransmitter functions encompass higher order cognitive processes, such as learning and memory, reward signalling, the control of endocrine function as well as locomotor control and sleep modulation. As a requirement for the diversity of physiological and behavioural roles, DA signalling is mediated by a complex interplay of molecular and cellular/neuronal elements. Impairments in this system lead to severe disease phenotypes, as it is the case for Parkinson's disease and schizophrenia. A more detailed understanding of normal physiological and pathophysiological processes is necessary in the development of new therapeutic strategies. However, the mammalian brain is complex; the employment of a "simpler" model to facilitate the deeper insight into the molecular processes is beneficial in this context. In this study, I have employed the fruit fly *Drosophila melanogaster* as a model organism to contribute to the understanding of the functions and elements of dopaminergic signalling. *Drosophila* possesses four DA binding receptors, DopR, DopR2, D2R and DopEcR. I have focused on the potential metabolic implications of DopR and D2R as well as their regulatory roles in activity and sleep behaviour.

DopR is broadly expressed in the central nervous system and in peripheral tissues of the fly. So far, it is known to hold central functions in memory formation and the modulation of arousal. By employing *DopR*-deficient flies (*dumb*<sup>2</sup> mutants) we have found several indications for an involvement of the receptor in metabolic functions in a potential sex-specific manner. *DopR*-deficient males show an excretory phenotype as well as impaired sleep behaviour and both traits were unchanged in females. However, potential reasons for this are currently unknown.

We have furthermore identified DopR as a possible upstream modulator of the diuretic hormone 44 (Dh44), which represents the fly homolog of the mammalian *corticotropin releasing hormone*. Via modulation of Dh44 signalling, DopR might be involved in water-retention mechanisms and Dh44-mediated activity enhancement.

D2R is the sole inhibitory DA binding receptor in the fly. D2R mutants possess a strong metabolic phenotype with an affected life expectancy and reduced body fat levels. A previous study identified a disturbance in the feeding initiation process, which, however, does probably not reduce food intake. The mutant might suffer from increased energy expenditure due to elevated activity levels (see below) but a general impairment in fat metabolism is conceivable.

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Moreover, D2R mutants show an unusual activity pattern. The flies are more active during the day and sleep more during the night than corresponding controls. The latter might represent a sleep rebound effect arising from increased daytime activity. However, the reduction in sleep during the day might be attributed to the absence of D2R-mediated inhibition of the DA arousal signal. In this regard, its expression in cells of the circadian clock system (LN<sub>vs</sub>) might be of central relevance. D2R is furthermore expressed in several light-receptive organs/cells that forward light information to the circadian clock system. However, potential receptor functions and contributions to the above described activity phenotype remain to be studied.

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## ZUSAMMENFASSUNG

Das biogene Amin Dopamin ist ein zentrales Signalmolekül, welches in vielfältigen physiologischen Prozessen beteiligt ist; konservierte Funktionen reichen von Wirbellosen bis zum Menschen. Die Hauptfunktionen als Neurotransmitter betreffen kognitive Prozesse wie Lernen und Gedächtnisfunktion, das Belohnungssystem, die Regulation des endokrinen Systems, Bewegungskontrolle und die Modulation des Schlafverhaltens. Der dopaminergen Signalübertragung liegt ein komplexes Zusammenspiel molekularer und zellulärer/neuronaler Elemente zugrunde, welches die Basis für die Diversität der Funktionen in Physiologie und Verhalten darstellt. Störungen in diesem System führen zu schweren Krankheits-Phänotypen, wie im Fall von Morbus Parkinson und Schizophrenie. Für die Entwicklung neuer therapeutischer Strategien ist eine genauere Kenntnis der normalen physiologischen und pathophysiologischen Prozesse des Dopaminsystems notwendig. Jedoch ist das Säugetiergehirn komplex und die Verwendung eines „einfacheren“ Modells von Vorteil, um einen tieferen Einblick in die molekularen Mechanismen zu erhalten. In der vorliegenden Studie wurde die Fruchtfliege *Drosophila melanogaster* als Modelorganismus verwendet, um zum Verständnis der Funktionen und Elemente des dopaminergen Systems beizutragen. In *Drosophila* sind vier dopaminbindende Rezeptoren bekannt, DopR, DopR2, D2R und DopEcR. Diese Arbeit konzentriert sich auf die potentiellen metabolischen Funktionen von DopR und D2R, sowie deren Beteiligung an der Kontrolle von Aktivität und Schlafverhalten.

DopR besitzt ein breites Expressionsmuster im zentralen Nervensystem und peripheren Organen in der Fliege. Bisher ist bekannt, dass der Rezeptor eine zentrale Bedeutung bei der Gedächtnisbildung innehat und an der Modulation des Wachheitszustands beteiligt ist. Durch Einsatz *DopR*-defizienter Fliegen wurden Hinweise auf metabolische Funktionen des Rezeptors gefunden, die sich möglicherweise geschlechtsspezifisch äußern. So zeigen die *DopR*-defizienten Männchen einen Phänotyp in der Exkretion und im Schlafverhalten, welcher in entsprechenden Weibchen nicht gefunden wurde. Die möglichen Gründe sind bisher nicht bekannt.

Des Weiteren konnte DopR als potentieller übergeordneter Modulator des Diuretischen Hormons 44 (DH44) identifiziert werden, welches das Fliegenhomolog des in Säugetieren vorhandenen *Corticotropin-releasing* Hormons darstellt. Durch die Modulation von DH44 könnte DopR an Wasserretentionsmechanismen beteiligt sein, sowie an einer durch DH44 vermittelten Aktivitätssteigerung.

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D2R ist der alleinige inhibitorische dopaminbindende Rezeptor in der Fliege. D2R-Mutanten zeigen einen klaren metabolischen Phänotyp durch eine beeinträchtigte Lebensspanne und reduzierten Körperfettgehalt. Eine vorangegangene Studie zeigte Störungen bei der Einleitung des Nahrungsaufnahmeprozesses, welche jedoch nicht zu verminderter Nahrungsaufnahme führen. Es ist möglich, dass die Mutante unter einem erhöhten Energieverbrauch leidet, welcher aus einem gesteigerten Aktivitätslevel resultieren könnte (s.u.), jedoch ist auch eine Störung des Fettstoffwechsels denkbar.

Darüber hinaus zeigen D2R-Mutanten ein ungewöhnliches Aktivitätsmuster. Im Vergleich zu entsprechenden Kontrollen zeigen die Fliegen am Tag eine gesteigerte Aktivität sowie eine Steigerung des Schlafs in der Nacht. Letzteres könnte einen Mechanismus darstellen, um die gesteigerte Tagesaktivität zu kompensieren. Die reduzierte Schlafmenge am Tag könnte mit dem Fehlen der normalerweise durch D2R vermittelten Inhibition des Dopamin-, „Wachsignals“ in Verbindung stehen. In diesem Zusammenhang könnte die Expression des Rezeptors in Zellen der endogenen Uhr (LN<sub>vs</sub>) eine zentrale Relevanz haben. D2R ist des Weiteren in mehreren Lichtempfangsorganen/-zellen exprimiert, welche Lichtinformation an die endogene zirkadiane Uhr weiterleiten. Potentielle Rezeptorfunktionen in diesen Systemen und die Beteiligung der Systeme am beschriebenen Phänotyp der Mutante müssen noch herausgestellt werden.

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# 1 INTRODUCTION

## 1.1 Dopamine as a neurotransmitter

Dopamine (DA; 3, 4-dihydroxyphenylethylamin) is a monoaminergic catecholamine. It is a bioactive molecule found from bacteria to plants and animals and might rather be considered as general “mediator” to cover all biological implications (Roshchina, 2010). For instance, DA and its precursor L-dopa have been isolated from different plant species and associated with diverse, yet in part uncertain functions. The study by Guidotti *et al.* (2013), in which DA was found to be released by the velvetbean and acting as allelochemical against neighbouring plants represents just one example.

However, at earlier times, the reason for DA existence was thought to be its role as precursor in the synthesis of norepinephrine. Only from the second half of the last century it was recognised as a major neurotransmitter in the mammalian brain. In 1957, Arvid Carlsson developed a method to detect DA in brain tissue and reported about highest concentrations in basal ganglia in the mammalian brain. For this discovery and his subsequent work on DA systems he was awarded the Nobel Prize for Physiology and Medicine in 2000 together with Paul Greengard and Eric Kandel. However, today, DA is primarily known as a central chemical modulator of the excitement state of nerve cells, acting at chemical synapses, and referred to as neurotransmitter. This function as messenger in the nervous tissue of animals is evolutionarily ancient with first evidence reported from different groups of cnidaria (Bielecki, Nachman and Garm, 2013; Chung and Spencer, 1996; Moroz and Kohn, 2016). Throughout the animal kingdom, CNS derived DA is associated with a variety of physiological and behavioural processes: associative learning, mood, motivation and the reward system, as well as hormone release. A major, potentially conserved, function is the role as modulator in motor circuits (see below).

### 1.1.1 Overview of dopamine synthesis and signal transmission

DA biosynthesis starts from the amino acid L-tyrosine, alternatively phenylalanine, which are usually supplied with dietary proteins. In the dopaminergic neuron, the enzyme tyrosine hydroxylase (TH) converts tyrosine to 3, 4-dihydroxy-L-phenylalanin (L-dopa), the rate-limiting step in the synthesis pathway. DA is finally generated from its precursor, L-dopa, by the dopa decarboxylase (DDC). Following synthesis, the neurotransmitter is stored in secretory vesicles

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at synapses, the cellular interaction sites of neurons. DA uptake into these storage organelles is mediated by an ATP-dependent substrate/H<sup>+</sup>-antiporter, the vesicular monoamine transporter 2 (VMAT-2; Erickson, Eiden and Hoffman, 1992) (Figure 1).

The DA release process from its synaptic vesicles is initiated upon arrival of an action potential at the presynaptic axon ending. Membrane depolarisation by sodium ions (Na<sup>+</sup>) causes the opening of voltage-gated calcium channels at the axon terminal and thus the influx of Ca<sup>2+</sup> ions. Calcium ions facilitate the fusion of transmitter vesicles with the presynaptic membrane leading to DA release (exocytosis) into the synaptic cleft and transmitter diffusion towards the surface of receiving cells. DA mediates its effects by binding to specific G protein-coupled receptors embedded in the postsynaptic membrane. A subsequent termination of the signal is usually achieved by Na<sup>+</sup>-dependent transmitter reuptake via the DA transporter (DAT) into the presynapse and recycling into vesicles (Kouzmenko, Pereira and Singh, 1997) or enzymatic conversion by monoamine oxidases (Yang and Neff 1974) and catechol O-methyltransferases (Axelrod and Tomchick 1958).

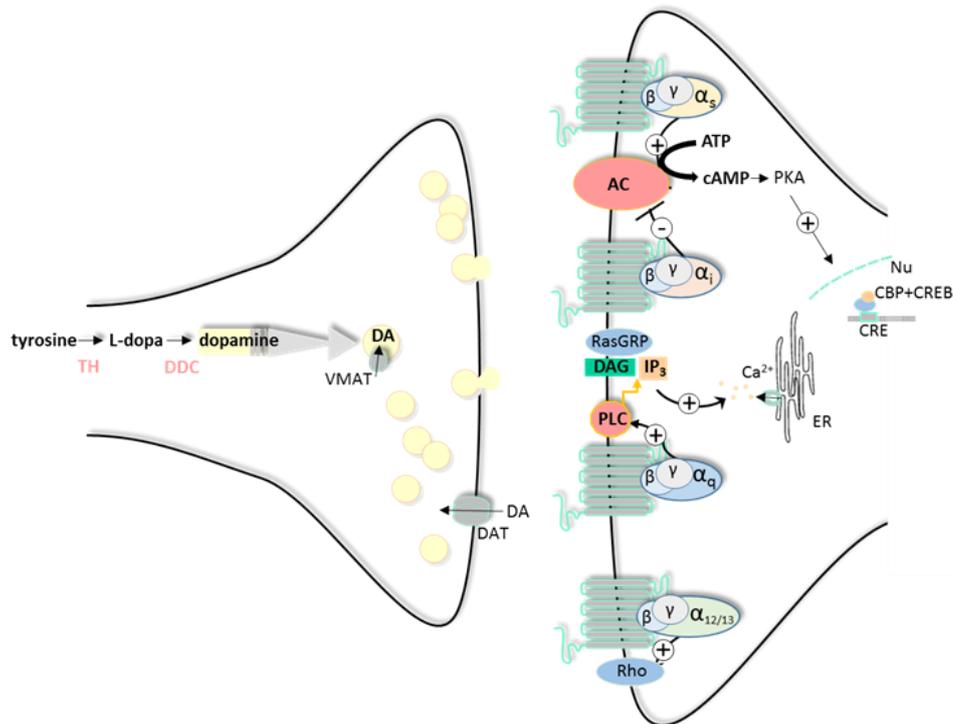
DA receptors are members of the rhodopsin-like family of metabotropic G protein-coupled receptors (GPCR) (Callier *et al.*, 2003; Vallone, Picetti and Borrelli, 2000). These receptors possess a heptahelical transmembrane region with alternating intra- and extracellular links between the helices, an extracellular amino (N-) terminus and an intracellular carboxy (C-) terminus. DA binding occurs via residues exposed by the transmembrane helices (Blenau and Baumann, 2001; Kalani *et al.*, 2004).

Upon transmitter binding, the receptor is activated, undergoes conformational change and transmits the signal to the inner of the cell. At the cytoplasmic site, the signal is received by a membrane- and receptor-associated heterotrimeric GTP-binding protein (G protein), which confers the specificity and type of further signal propagation. G proteins are composed of 3 subunits, denoted as G $\alpha$ -, G $\beta$ - and G $\gamma$ -subunit. The  $\alpha$ -subunit binds a guanosine diphosphate (GDP) and forms a tight association with the G $\beta\gamma$ -subunit. However, the binding of a ligand, in this case DA, to the GPCR activates the receptor's function as guanine nucleotide exchange factor (GEF), which in turn causes the release of GDP from G $\alpha$  and exchange to guanosine triphosphate (GTP). The heterotrimeric protein complex dissociates into the G $\alpha$  and G $\beta\gamma$ -subunits, which further act on downstream membrane-associated effectors. Finally, the intrinsic GTPase activity of G $\alpha$  terminates the signal and brings back the starting condition.

### 1.1.2 Overview of dopamine receptor associated messenger systems

Based on the sequence similarities of  $\alpha$ -subunits and accompanying functional similarities, G proteins are assigned into 4 classes, each comprising different numbers of family variants:  $G\alpha_s$ ,  $G\alpha_i$ ,  $G\alpha_q$ , and  $G\alpha_{12/13}$  (Figure 1; McCudden *et al.*, 2005; Strathmann and Simon, 1990, 1991). (1) Stimulatory G proteins containing  $G\alpha_s$  stimulate adenylate cyclase (AC), a membrane-associated enzyme, to generate the second messenger cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). cAMP pursues the signalling pathway and interacts with a variety of effectors, e.g. the protein kinase A (PKA). PKA further induces signalling cascades that might finally lead to transcription initiation of target genes containing a cAMP-response element (CRE) as regulatory sequence. (2) G proteins containing  $G\alpha_i$  interact with AC but block its enzymatic activity and thus lower the local cAMP concentration. These G protein complexes are of inhibitory function upon receptor stimulation (Taussig *et al.*, 1994). (3)  $G\alpha_q$  containing G proteins do not interact with AC but are using an alternative second messenger system.  $G\alpha_q$  stimulates phospholipase C (PLC) to hydrolyse the membrane-anchored phosphatidylinositol 4, 5-bisphosphate (PIP<sub>2</sub>), thereby generating inositol 3-phosphate (IP<sub>3</sub>) and diacylglycerol (DAG) (Mizuno and Itoh, 2009). The soluble IP<sub>3</sub> diffuses through the cytoplasm and opens Ca<sup>2+</sup> channels at the endoplasmic reticulum to allow calcium ion influx into the cytosol. The signal might now be further propagated by Ca<sup>2+</sup> itself, as it represents another second messenger (Rasmussen *et al.*, 1976). However, due to its lipophilic properties, DAG stays in the cell membrane from where it interacts with downstream effectors, such as RasGRP (GEF activating the monomeric G protein Ras; Lorenzo *et al.*, 2000) and protein kinase C (PKC; Kishimoto *et al.*, 1980). (4)  $G\alpha_{12/13}$  represent a fourth class of  $G\alpha$  subunits. Signalling via this type of  $G\alpha$  has been associated with a variety of physiological processes.  $G\alpha_{12/13}$  activates Rho GTPase in the family of small G proteins (Hart *et al.*, 1998; Suzuki *et al.*, 2003) and associated downstream cascades.

Current research on GPCR covers different complexity mechanisms that underlie physiological flexibility. For instance, GPCRs have been found to occur in homo- and heterodimers and oligomers. The occurrence of these receptors in associations with other receptors has been demonstrated to influence receptor function and thus might be of crucial medical relevance. Oligomerisation with mutant receptor partners might alter associated signalling pathways leading to cellular dysfunction. Moreover, pharmaceuticals acting as receptor agonists might be affected in their mechanism of action (Lee *et al.*, 2000; Łukasiewicz *et al.*, 2010; Milligan, 2009).



**Figure 1.1 | Overview of DA synthesis and signalling via GPCRs and different types of G proteins.** DA is synthesised from the amino acid tyrosine in 2 enzymatic steps and sequestered into vesicles via the vesicular monoamine transporter (VMAT) within the presynaptic axon ending. Upon DA cell activation and action potential propagation, DA is released into the synaptic cleft and diffuses to bind to DA specific G protein-coupled receptors (GPCRs) located on the postsynaptic neuron. Intracellular signalling is continued depending on the type of G protein associated with the GPCR. (1)  $G\alpha_s$  stimulates the adenylate cyclase (AC) to generate the second messenger cAMP (cyclic adenosine monophosphate). Further downstream signalling cascades typically involve the protein kinase A (PKA), which modulates the transcription of genes bearing a cAMP response element (CRE) in their regulatory region. (2)  $G\alpha_i$  inhibits AC activity thereby lowering the cellular cAMP level. (3)  $G\alpha_q$  stimulates phospholipase C (PLC) to hydrolyse the membrane-anchored phosphatidylinositol 4, 5-bisphosphate, thereby generating the second messengers diacylglycerol (DAG; further interacting with RasGRP) and inositol 3-phosphate (IP<sub>3</sub>; stimulates release of Ca<sup>2+</sup> from the endoplasmic reticulum (ER)), as well as Ca<sup>2+</sup>. (4)  $G\alpha_{12/13}$  interacts with RhoGTPase. The reuptake of DA into the presynapse by the DA transporter (DAT) represents a mechanism to terminate the signal. Nu – nucleus, CBP – CREB binding protein, CREB – cAMP response element binding protein. Figure contains elements from the PowerPoint toolkit by motifolio.com.

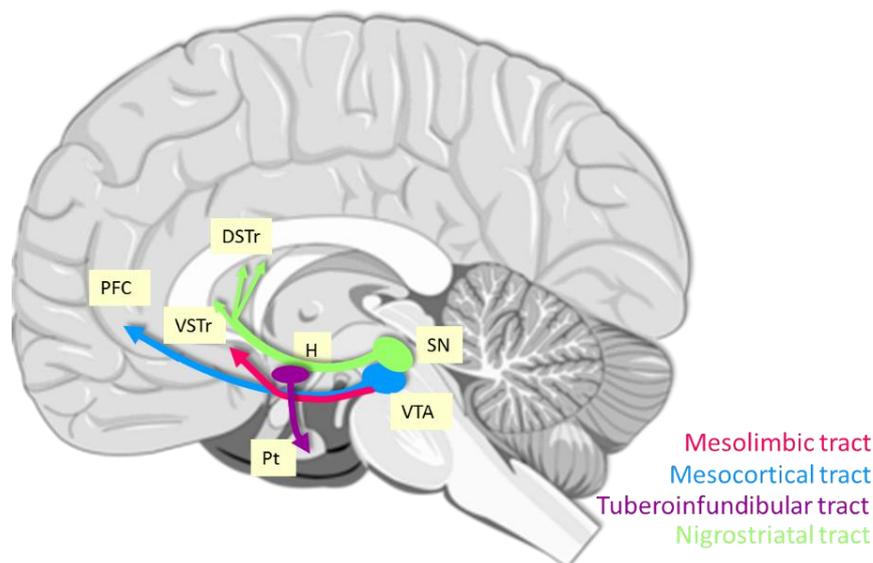
However, based on the nature of interaction with AC, DA receptors are classified into two groups. (1) D<sub>1</sub>-class receptors are coupled to G<sub>s</sub> leading to a rise in cellular cAMP levels. (2) D<sub>2</sub>-class receptors exert inhibitory effects on AC or do not interact with the enzyme (G<sub>i</sub> coupling) (Kebabian, Beaulieu and Itoh, 1984; Kebabian and Calne, 1979). D<sub>1</sub>- and D<sub>2</sub>-class receptors share the ability to bind DA, however, both receptor groups appear unrelated in molecular phylogenies (Callier *et al.*, 2003). Moreover, genetic differences lead to different

levels of diversity among the family members at least in vertebrates, as D<sub>1</sub>-like receptors, unlike D<sub>2</sub>-class receptors, do not possess introns. Humans express 5 distinct DA receptors of which D<sub>1</sub> and D<sub>5</sub>/D<sub>1b</sub> belong to the D<sub>1</sub>-like receptor class and D<sub>2-4</sub> are grouped within D<sub>2</sub>-like receptors. Please note that the concept of D<sub>1</sub>-like and D<sub>2</sub>-like classification in today's view might only provide a simplified overview, as accumulating molecular data rather indicate higher levels of complexity (Andersen *et al.*, 1990).

### 1.1.3 The neuronal dopaminergic system in humans – Anatomy and consequences of associated malfunctions

DA is involved in diverse body functions, such as the control of voluntary movements, the creation of emotions and motivation, the reward system, cognitive processes and hormonal control. DA imbalances or pathway dysfunction lead to mental, hormonal and locomotor deficits.

In the human brain, DA neurons are organised in dopaminergic projections referred to as pathways. Somata of DA synthesising cells cluster to specific brain regions and extend their axonal processes towards particular sites at which DA release takes place. In general, 9 dopaminergic pathways are distinguished, of which the 4 key projections that encompass 400 000 – 600 000 DA neurons are introduced in the following paragraph (Figure 2; Björklund and Dunnett, 2007).



**Figure 1.2 | Dopaminergic projections in the human brain.** 4 key dopaminergic pathways are distinguished: (1) The nigrostriatal tract projects from the Substantia nigra (SN) to the dorsal striatum (DSTr) and is involved in movement control. (2) + (3) The mesocorticolimbic pathway originates in the ventral tegmental area and projects to the ventral striatum (mesolimbic pathway) and the prefrontal cortex (mesocortical pathway). It is involved in the rewarding system and executive functions. (4) The tuberoinfundibular pathway projects from the hypothalamus to the pituitary gland to control hormone (prolactin) release. After <http://thebrain.mcgill.ca>.

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(1) Projections of the nigrostriatal pathway are built by >70 % of all the DA neurons. They originate in the midbrain *Substantia nigra pars compacta* and extend to the dorsal striatum (caudate nucleus and putamen) of the basal ganglia. Here, DA is involved circuits that function in operant conditioning, related decision-making to select action based on sensory information, motivation and cognitive evaluation (Balleine, Delgado, and Hikosaka 2007; Becker-Hapak *et al.* 2004), motor control and further contribute to the reward system. Associated diseases often express impaired movement and cognitive ability, as it applies for Parkinson's (PD) and Huntington's disease. Whereas for the latter, among other factors, a DA release imbalance in the nigrostriatal pathway is assumed to promote disease onset (André, Cepeda and Levine, 2010), the pathway is directly affected in PD, as its best known indicator is the degeneration of DA producing cells in the *Substantia nigra pars compacta* (Minati *et al.*, 2007). However, PD patients suffer from motor impairments that include tremor, slow movements (bradykinesia), disability to control/initiate movement (akinesia), as well as cognitive and psychological impairments and further symptoms affecting peripheral musculature. The disease origin is still unknown but genetic and environmental factors are speculated.

The mesocorticolimbic pathways originate in the ventral tegmental area (VTA). From here, the axonal projections split up (2) to the ventral striatum (*Nucleus accumbens*) representing the mesolimbic pathway with major roles in the reward system and the creation of motivation and (3) to the prefrontal cortex to build the mesocortical pathway, which is involved in cognition attention and other executive functions. Both pathways are implicated in the development of addictions (Adinoff, 2004), depression (Nestler and Carlezon, 2006) and schizophrenia (Weinstein *et al.*, 2017). (4) The tuberoinfundibular pathway consists of projections that extend from the hypothalamus to the pituitary gland to control the release of the hormone prolactin.

## 1.2 Dopamine in invertebrates

The advent of nervous tissue was necessarily accompanied by the evolution/occupation of appropriate chemical messenger systems (neurotransmitters and receptors), however, to date, the molecular and structural make-up remains unresolved for most invertebrate phyla. Thus, conclusions on neurotransmitter systems, including the dopaminergic structures and the physiological relevance of DA, are incomplete especially for cnidarians, molluscs, annelids and

plathelminthes. Traces of DA (meaning the substance itself and its metabolising enzymes) have been detected in almost all phyla, accompanied by evidence for its modulatory role in motor circuits (Barron, Søvik and Cornish, 2010). However, since exceptions are known, e.g. ctenophores do not employ DA as neurotransmitter (Moroz *et al.*, 2014), further analyses are needed to assess the universal relevance. More clear data is available from “ectdysozoans”. The nematode *Caenorhabditis elegans* possesses 8 dopaminergic neurons that underlie the mechanosensation of food (bacteria) and directly provoke the slowdown of locomotion to benefit food consumption (Barron *et al.*, 2010; Sawin *et al.*, 2000). This “simple” motor circuit might represent a very basic situation that has evolved to different complexity levels in other animal groups.

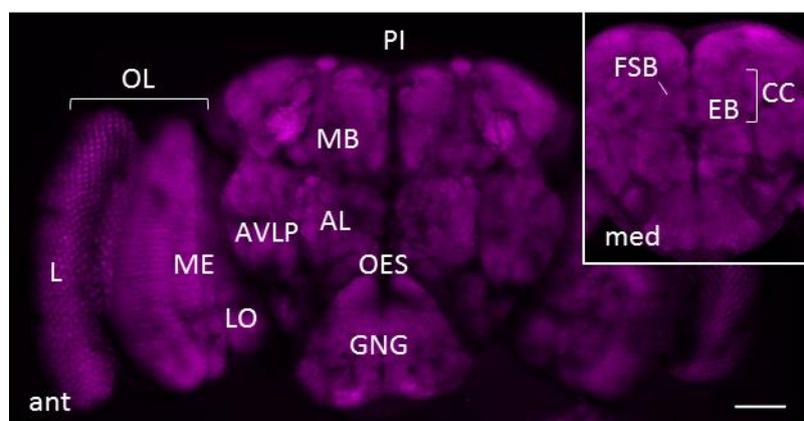
Among the invertebrates, DA is best studied in insects, especially in *D. melanogaster*. In the fly, it has been found to be involved in motor circuits acting directly on neuromuscular junctions (Cooper and Neckameyer, 1999) and, presumably, modulatory in higher “locomotor control centres” (e.g. Riemensperger *et al.*, 2011). It has been shown to mediate arousal under different conditions (Andretic, Van Swinderen and Greenspan, 2005; Kim *et al.*, 2016; Kume *et al.*, 2005), to be involved in learning and memory (Berry *et al.*, 2012; Liu *et al.*, 2012), the regulation of feeding (Marella, Mann and Scott 2012) and to be involved in stress responses and aggressive behaviour (Alekseyenko, Chan and Kravitz, 2013; Argue, Neckameyer and Wend,i 2013).

### **1.3 The nervous system of *D. melanogaster***

Fruit flies possess a central and peripheral nervous system (C/PNS). The CNS is comprised of a brain (syncerebrum), located in the head capsule, and connected to a ventral ganglionic cord (ventral nerve cord, VNC) that extends through the thorax. According to the arthropod bauplan, the brain consists of 2 major parts, the supra- and suboesophageal ganglion, which are perforated by the oesophagus. The supraoesophageal ganglion developed from 3 coalesced ganglia, the proto-, deuto- and tritocerebrum. Moreover, the VNC is composed of 3 coalesced thorax ganglia and one abdominal ganglion (highly coalesced abdominal ganglia, formerly segmental). On the cellular level, axonal processes originate from the neuronal cell body and form the neuropil areas together with their synaptic connections. Neuronal circuits comprise e.g. afferent sensory neurons that deliver environmental input signals from sense organs, such as the antennae or compound eyes, and efferent motor neurons, which extend axons towards the periphery

and directly connect with muscle cells to control contractions. Different neuropil areas are interconnected by interneurons. Besides nerve cells, the CNS consists of 10% nonneuronal glia cells that enwrap the neurons (Kremer *et al.*, 2017). Glia cells support the development of neurons and synaptic connections, deliver nutrients, constitute the blood-brain barrier and probably even directly serve in neurotransmission.

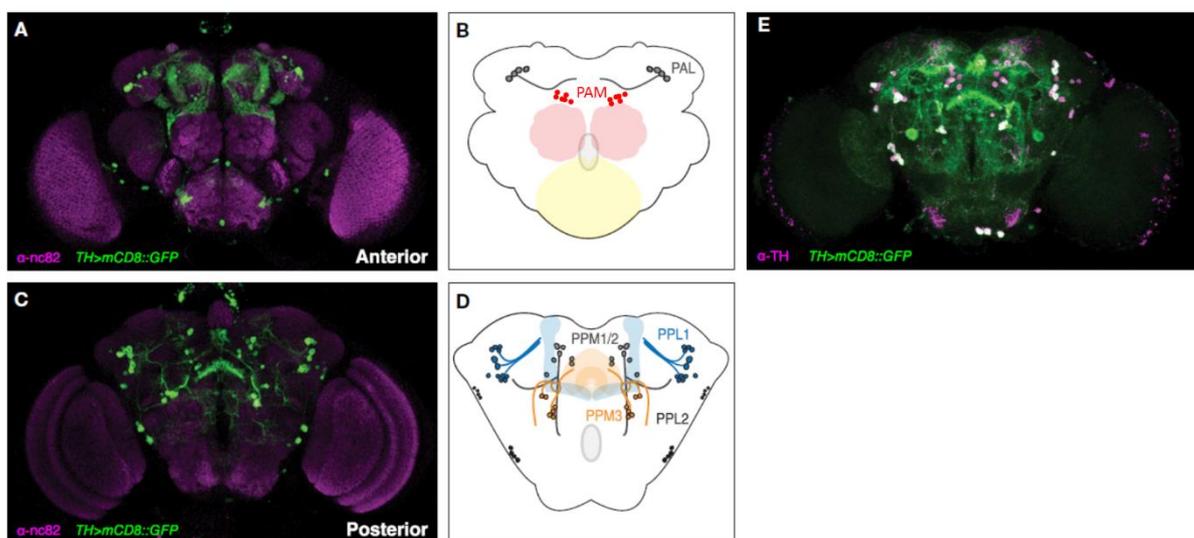
The adult fly brain can be subdivided into various bilateral synaptic neuropil areas (Figure 3; Ito *et al.*, 2014; <http://www.virtualflybrain.org>). Among the most prominent areas are the antennal lobes (AL), which receive input from the antennae via olfactory receptor neurons; the mushroom bodies (MB), which are associated with higher order brain functions, learning and memory, are subdivided into calyx, pedunculus, and  $\alpha$ -, $\beta$ - and  $\gamma$ -lobes; the central complex (CC), which integrates spatial information and controls motor behaviour, encompasses the ellipsoid body (EB, a doughnut-shaped structure), the fan-shaped body (FSB), the noduli and the protocerebral bridge; the optic lobe (OL) is comprised of the medulla (ME) and accessory medulla, lobula complex and lamina (L) and the gnathal ganglia (GNG), consisting of sensory centers of pharynx and mouthparts. An additional region is represented by the *pars intercerebralis* (PI), the neuroendocrine centre, which contains neurosecretory cells that synthesise various hormones.



**Figure 1.3 | Main neuropil areas of the *Drosophila* adult brain.** Neuropils have been visualised by anti-NC82 antibody staining. The most prominent neuropils are represented by the mushroom bodies (MB), antennal lobes (AL), gnathal ganglia (GNG), central complex (CC; inset; EB – ellipsoid body, FSB – fan-shaped body) and the optic lobe (OL; ME – medulla, LO – lobula, L – lamina). AVLP – anterior ventrolateral protocerebrum; OES – oesophageal foramen; PI – *pars intercerebralis*; ant – anterior view; med – medial plane view. Scale bar = 50  $\mu$ m.

### 1.3.1 Structural anatomy of the dopaminergic system

As in mammals, DA is produced in a 2-stage enzymatic reaction in insects. The first and rate-limiting step in DA synthesis is carried out by TH, which hydroxylates tyrosine to generate the direct DA precursor L-dopa. In *Drosophila*, TH is encoded by the *pale* locus (*ple*) (Neckameyer and White, 1993). The presence of TH is thus considered as diagnostic marker for the DA synthesis ability of cells. Based on TH expression patterns, the cellular distribution of dopaminergic cells has been characterised in the CNS. The following description applies to the adult *Drosophila* brain and is based on the studies by Friggi-Grelin *et al.* (2003) and White, Humphrey and Hirth (2010). Cell bodies of DA producing neurons group into 6 different clus-



**Figure 1.4 | Dopaminergic cell clusters in the adult brain of *Drosophila*.** GFP expression (green) was targeted to the sites of th promoter activity (TH>mCD8::GFP) by the use of the GAL4/UAS system. Brains were counterstained with  $\alpha$ -NC82 antibody to visualise neuropils. (A) shows the anterior clusters PAM and PAL (scheme in (B)). (C) shows the posterior clusters PPL1, PPL2, PPM1/2 and PPM3 (scheme in (C)). To verify the driver line (TH-GAL4) accuracy, brains were counterstained with  $\alpha$ -TH antibody (E). Taken from White *et al.* (2010), modified in (B).

ters, which consist of different numbers of cell bodies and project towards different functional regions in the brain (Figure 4). The two clusters that are located to the anterior brain, are the dorsolateral anterior protocerebral cluster (PAL), of which projections extend to different regions in the protocerebrum, and the dorsomedial anterior protocerebral cluster (PAM). All other clusters are located posteriorly. However, the clusters dorsolateral posterior protocerebral 1 and 2 (PPL1 and PPL2) are situated at the lateral sites of the brain and project ventrally and medially and innervate different regions of the mushroom bodies (Mao and Davis, 2009). The dorsomedial posterior protocerebral clusters 1 and 2 (PPM1/2) target broad regions in the ventrolateral protocerebrum. Furthermore, the central complex is innervated by DA cells of the dorsomedial posterior protocerebral cluster 3 (PPM3).

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### 1.3.2 Dopamine receptor expression patterns and functions

*Drosophila* possesses 4 DA binding receptors: Dopamine 1-like receptor 1 (DopR, DUMB; CG9652), Dopamine 1-like receptor 2 (DopR2, DAMB; CG18741), Dopamine/Ecdysteroid receptor (DmDopEcR; CG18314) and Dopamine 2-like receptor (D2R; CG33517). The following paragraphs provide an overview on receptor expression profiles and functional implications.

#### 1.3.2.1 The Dopamine 1-like receptor 1

DopR was the first of the fly's DA receptors that has been isolated and biochemically and pharmacologically characterised. The comparison of its secondary structure with vertebrate counterparts revealed conserved regions within the 7 transmembrane domains. However, other structural features, e.g. the intracellular G protein attachment site, as well as pharmacological characteristics appeared unrelated to corresponding vertebrate DA receptors. Nevertheless, in cell culture systems, it could be demonstrated that DopR mediates the generation of cAMP upon DA exposure and the receptor was thus classified as D<sub>1</sub>-like receptor (Gotzes, Balfanz and Baumann, 1994; Sugamori *et al.*, 1995).

Dop R expression has been analysed on the transcript and protein level, as well as by transgenic reporters. In situ hybridisation patterns of fly embryos indicated the presence of the receptor mRNA as maternal transcript at the syncytial blastoderm stage and *dopr* transcription throughout embryogenesis (Sugamori *et al.*, 1995). However, the function of this early transcript presence, e.g. a potential developmental role, remains unknown. Kim *et al.* (2003) generated an antibody against the third cytoplasmic loop of the DopR protein, as this region was determined as unique structural element (Sugamori *et al.*, 1995). Immunohistochemical analyses of direct DopR immunoreactivity (IR) revealed developmental stage specific expression patterns in larval and adult CNS. Generally, receptor IR has been detected in the central brain and VNC of larvae and adults. However, in the brain, only the strong labelling of the MB neuropil appeared consistent from larvae to adults, as well as the segmental pattern of somata in the thoracic and abdominal ganglia of the VNC (Kim *et al.*, 2003). In addition, in the adult brain, strong IR signal was detected in all substructures of the CC but not the PB (Kahsai *et al.*, 2012). Furthermore, DopR immunoreactive somata were observed in the GNG and in the posterior dorsal brain, the latter of which might represent neurosecretory cells (Kim *et al.* 2003; Stephano, 2013). Gotzes *et al.* (1994) additionally found high expression in the OL. By using targeted expression of a GFP reporter further DopR positive cells could be identified in peripheral tissues. High levels of GFP signal were detected in larval salivary glands, fat body (additionally

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supported by direct IR), enteroendocrine cells of the gut and the corpus allatum (direct IR) (El-Kholy 2010; N.E. Gruntenko, Laukhina, and Rauschenbach 2012; Stephano 2013).

DopR has been demonstrated to take part in a variety of behavioural and physiological processes in the fruit fly. Based on its prominent expression in the MB (see above), extensive studies revealed DopR to be crucial in creating associations between stimuli and rewarding or aversive perceptions. The receptor was assigned a critical role in classical appetitive and aversive olfactory learning, whereas for the latter it could be shown that DopR transmits the DA signals via the MB  $\gamma$ -neurons (Kim, Lee and Han 2007; Liu *et al.*, 2012; Qin *et al.*, 2012; Selcho *et al.*, 2009). Furthermore, the receptor mediates appetitive and aversive visual conditioning; this process uses cellular routes that partially correspond these used for olfactory conditioning (Vogt *et al.*, 2014).

Another behaviour mediated by DopR is sleep. Knockout flies showed elevated levels of total sleep per day arising from an increase in longest and average sleep bout duration and corresponding to increased sleep-wake arousal (Lebestky *et al.*, 2009). This indicates that DA exerts its wakefulness promoting effect via DopR. Ueno *et al.* (2012) could assign the receptor's expression site in the dorsal fan-shaped body (dFSB) to be responsible for the dopaminergic control of sleep. Liu *et al.* (2012) also found the DopR positive neurons in the dFSB to promote wakefulness. Since dopaminergic projections modulating sleep and arousal converge at this site of the CC, they propose a switch point function at which arousal centres modulate sleep-promoting centres. DopR is further involved in environmentally provoked arousal, which is, however, transmitted by distinct cellular pathways than endogenously triggered arousal (Lebestky *et al.*, 2009). The study suggests that a DA signal transmitted by DopR is acting inhibitory in startle-induced arousal. Moreover, the receptor might be indirectly associated with sleep regulation during ageing via IIS/TOR (insulin/insulin-like growth factor signalling/target of rapamycin) (Metaxakis *et al.*, 2014) and drug- (caffeine-) induced arousal (Andretic *et al.*, 2007).

Besides the above mentioned functions, DopR has been demonstrated to be important in the control of hind leg grooming behaviour (Pitmon *et al.*, 2016), in the stimulation of locomotor activity upon ethanol exposure (Kong *et al.*, 2010), in dopaminergic stress response circuits with a potential sex-specific importance (Argue, Neckameyer and Wendi, 2013), at different phases of the stereotypic courtship behavioural sequence (Chen *et al.*, 2012; Keleman *et al.*, 2012) and in juvenile hormone metabolism (Gruntenko, Laukhina and Rauschenbach, 2012;

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Rauschenbach *et al.*, 2011). In addition, Alekseyenko, Chan and Kravitz (2013) found indications for a role of DopR in aggressive behaviour.

### 1.3.2.2 Dopamine 1-like receptor 2

The second isolated DA receptor gene appeared structurally distinct from DopR and related mammalian DA receptors. However, GPCR characteristics, such as the 7 transmembrane regions, as well as potential G protein binding residues were detected. Pharmacological studies in cell culture systems could substantiate the intracellular generation of cAMP upon DA exposure and the receptor was grouped into the D<sub>1</sub>-like class. RNA based expression analysis revealed predominant expression in the head and only minor transcript amount or no detectable signal for the periphery or trunk (Feng *et al.*, 1996; Han *et al.*, 1996). These results were verified with a specific antibody targeting the third cytoplasmic loop of the receptor. Using direct IR, highest expression levels in the MB axonal tracts were visualised, representing the major site of expression for this receptor; based on this finding the receptor was assigned the common name DAMB ('dopamine receptor in mushroom bodies'). Weak IR signal was detected in the CC. The expression sites were mostly in accordance with the pattern of a GFP reporter, of which expression was targeted to *damb* transcribing cells. However, since the usage of a transgenic reporter with subsequent antibody staining represents a strong enhancement of the signal, additional potential DAMB positive cells were detected e. g. in the GNG, ME and LO of OL and VNC (direct IR was shown by (Cassar *et al.*, 2014)) (El-Kholy, 2010). Furthermore, GFP signal was detected in peripheral tissues, such as the enterocytes of mid and hind gut and the tracheal branches (El-Kholy, 2010; Stephano, 2013).

Several functional contexts have been reported for DAMB. Due to its high expression level in the MB, an involvement of DAMB in memory processing was assumed. In their experimental series, Berry *et al.* (2012) found the knockout mutant of the receptor to be impaired in electric shock avoidance behaviour at commonly used voltages. However, most importantly, they demonstrated a potential role of DAMB in the process of forgetting. While DopR acts in parallel in the MB in the aversive memory acquisition process (see above), it is believed that DAMB is involved in active forgetting of the acquired "knowledge" at least in conventional fly conditioning regimes. In accordance to this, the DAMB mutant shows enhanced memory ability. These results might be supported by another study on the  $\alpha$ -synuclein induced model of Parkinson's disease. The authors tested the detrimental effect of sleep deprivation on degenerating

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DA neurons and found an increased level of *dopr* and *damb* mRNA in sleep-deprived  $\alpha$ -synuclein expressing flies. A blocking of both receptors abolished the short and long term memory deficits that usually result from sleep-deprivation in these flies (Seugnet *et al.*, 2009).

DAMB expression was observed to increase in an age-related manner, which appears associated with the decrease in resistance to paraquat (PQ), a herbicide causing oxidative stress in the organism. Flies depleted in the receptor showed higher survival rates under PQ exposure than corresponding controls. This suggests that the receptor is involved in age-dependent alterations in susceptibility to oxidative stress (Cassar *et al.*, 2014). Relating to this finding, the receptor has been demonstrated to be involved in stress response circuits of starvation and oxidative stress (Argue *et al.*, 2013).

Furthermore, DAMB has been demonstrated to be essential in the developing fly, especially during metamorphosis. Flies diminished in the receptor could not develop into imagos or were affected in unfolding of wings or melanisation. Transcriptomic analysis suggested a key inhibiting/modulating effect of DAMB on metamorphosis genes (Regna *et al.*, 2016).

#### 1.3.2.3 Dopamine 2-like receptor

The *Drosophila* D2R has been the first insect D<sub>2</sub>-like receptor that was isolated and characterised. Homology comparisons with mammalian D<sub>2</sub>-like receptors could identify highly conserved regions, such as the heptahelical transmembrane region. Heterologous receptor expression could further reveal its coupling to G<sub>i</sub> as, upon application of DA, an experimentally (forskolin) induced elevated cAMP level was significantly lowered. This finding substantiates the classification of this receptor as D<sub>2</sub>-like. Binding affinity experiments revealed DA as most potent ligand, however, interestingly, other biogenic amines, such as serotonin and tyramine but not octopamine also caused significant receptor stimulation. Further studies on the potential physiological relevance of this finding are needed at this point (Hearn *et al.*, 2002).

D2R expression was analysed on the protein level by the use of a polyclonal antibody targeting the conserved third extracellular loop of the receptor (Draper *et al.*, 2007). IR has been found in processes of larval brain hemispheres, yet without corresponding somata. In the VNC, a segmental pattern of somata and processes (“fishbone-like”) was detected throughout the thoracic and abdominal segments. In the adult CNS, processes extended within the brain hemispheres and distinct somata could be identified. Two clear cell bodies were located in the dorsolateral protocerebrum. Additional somata were described in the OL, specifically in L and ME and in

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the anterior ventral brain within the GNG, in MB, as well as in the PB within the CC (Alekseyenko *et al.*, 2013; Draper *et al.*, 2007; El-Kholy, 2010; Stephano, 2013). In the adult VNC, D2R positive somata appear arranged in groups of 4 pairs in each thoracic segment and in an undefined number in the abdominal ganglion. GFP reporter based analyses and direct IR could furthermore reveal receptor expression in peripheral organs. Labelled cell bodies were found in the foregut and two regions of the larval midgut (ventriculus and posterior midgut with processes extending towards hindgut and lumen), trachea, salivary glands, Malpighian tubules (probably principal cells), the fat body and *corpus allatum* (El-Kholy 2010; Gruntenko *et al.*, 2012). GFP reporter based analyses could further identify the D2R positive cells in the adult midgut as enteroblasts and intestinal stem cells (Stephano, 2013). Qi and Lee (2014) demonstrated the necessity of D2R as autoreceptor and in postsynaptic locations in the MB for aversive and appetitive olfactory learning in larvae. Furthermore, the receptor has been found to underlie anesthesia-resistant memory, a specific form of consolidated memory, which can, at least in part, be ascribed to its localisation in MB Kenyon cells (Scholz-Kornehl and Schwarzel, 2016).

Draper *et al.* (2007) tested the effect of diminished D2R levels on locomotor behaviour. Locomotor levels of panneuronally D2R-depleted flies were impaired, as total activity counts, the amount of time active, as well as the number of activity/rest bouts were decreased. The phenotype could be rescued by application of bromocriptine, a well-known D2R agonist and established anti-Parkinson treatment. Interestingly, transcriptomic data has shown receptor expression in large lateral-ventral neurons, which belong to the clock neuron system. It is believed that these cells mediate the wake-promoting effect of DA, however, this effect was inhibited by light. D2R has been suggested to take part in a sophisticated regulatory mechanism that suppresses a DopR-dependent cAMP signal, thereby promoting a rest state under light-dark circadian cycles. Light would stimulate or activate D2R activity with the requirement of light-dependent modulation of *d2r* gene expression or direct stimulation of receptor activity or downstream signalling factors by light (Shang *et al.*, 2011).

Furthermore, D2R is involved in sugar-sensing circuits. It contributed to the choice process in females, in which the value of a sugar substrate is determined to decide upon a preference substrate for egg-laying (Yang, He and Stern, 2015). In addition, in the GNG, the receptor receives input from a single dopaminergic neuron (TH-VUM, for TH positive, ventral unpaired medial neuron) and has been shown to transmit the signal that leads to proboscis extension (Marella, Mann and Scott, 2012).

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D2R has also been found to be neuroprotective against MPP<sup>+</sup> (neurotoxin, blocks ATP generation in mitochondria of dopaminergic cells) in cell culture. Upon activation of D2R located as autoreceptor in DA producing cells, the initiated signalling cascade lowers the excitability of the cell, which, by an unknown mechanism, protects the cell against MPP<sup>+</sup>-induced damage (Wiemerslage *et al.*, 2013). This finding represents a promising principle in the development of new therapeutic strategies of Parkinson's disease in humans.

Beyond the above mentioned functions, D2R has been associated with a sex-specific stress response circuit (Argue *et al.*, 2013), a possible role in aggressive behaviour (Alekseyenko *et al.*, 2013) and the control of endocrine factors, such as an inhibitory effect on juvenile hormone synthesis in the *corpus allatum* (Gruntenko *et al.* 2012a; Gruntenko *et al.* 2012b).

#### 1.3.2.4 Dopamine/Ecdysteroid receptor

The DopEcR does not represent a typical DA receptor. Srivastava *et al.* (2005) isolated the receptor protein and found structural similarities to GPCR, e.g. the 7 transmembrane domains (cell surface receptor), and potentially conserved residues known to interact with catecholamines. They could show selective binding of DA to this receptor, which also led to a substantial rise in cAMP in the cells and subsequent stimulation of the phosphoinositide 3-kinase pathway. Based on its unusual pharmacology reminiscent of some vertebrate receptors binding to steroids, other potential ligands were tested. Ecdysone and 20-hydroxyecdysone, two insect steroid hormones involved in moulting and metamorphosis, were also found to bind with high affinity to this receptor. Upon steroid binding, the signalling mode might be inhibitory on the cAMP signal originating from DA mediated induction.

RNA-based analyses of expression pattern revealed developmental stage specific regulation of DopEcR transcription. At early embryonic stages, receptor transcript was detected in the gut and salivary glands. In addition, the *DopEcR* transcript was found in the developing CNS up to the third larval instar but not at later stages. GFP reporter pattern, targeted to DopEcR positive cells, uncovered prominent expression in the MB in adult fly brains. Here, the receptor is involved in particular associative learning processes, as it has been shown for the development of experience-dependent courtship memory in males (Ishimoto *et al.*, 2013). Additional GFP signal was detected in the AL, CC, GNG, as well as in the VNC.

DopEcR is present in gustatory receptor neurons, where, under starvation conditions, it transmits DA signals to the sensory neurons to enhance sugar sensitivity along with the proboscis

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extension reflex (Inagaki *et al.*, 2012). Moreover, the receptor seems to be involved in an evolutionarily conserved response circuit to ethanol exposure and mediates ethanol-induced sedation (Petrucci *et al.*, 2016).

## 1.4 The fruit fly and its versatility as a model organism

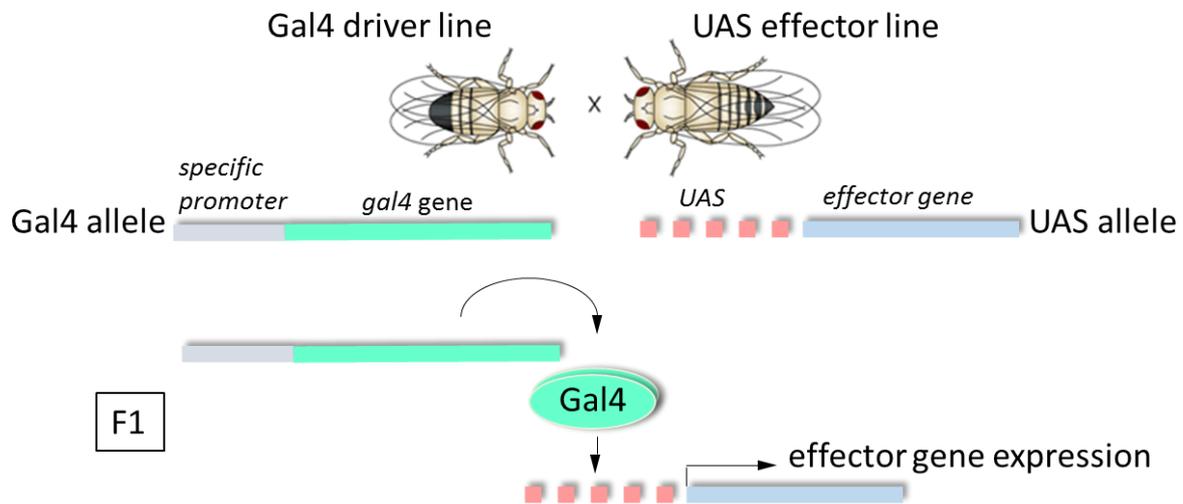
Nearly 100 years after its first scientific description by J. W. Meigen (“Systematic description of the known European two-winged insects”, 1830) the fruit fly’s career as model in genetics was founded in the laboratory (“The Fly Room”) of T. H. Morgan at Columbia University. Morgan discovered, together with his students A. H. Sturtevant and C. B. Bridges, that elements of heredity are located in arrays of a certain order on chromosomes and they created the first chromosomal maps. Morgan was later awarded the Nobel Prize in 1933 for this work. Since then, *Drosophila melanogaster* has become a famous model in diverse biological issues, encompassing the fields of developmental biology, morphology, cell and molecular biology, systematics, evolution and genetics and physiology, neuro- and behavioural biology. Its popularity is attributable to the convenient, cheap and easy, culturing demands, the short generation time, as well as its genetic amenability with elaborated sequence data and manipulation tools.

*D. melanogaster*’s original natural home is the sub-Saharan Africa, from where it spread to all continents (except Antarctica) within the last 15 000 years (Hales *et al.*, 2015). Its common name “fruit” or “vinegar fly” describes the natural feeding behaviour on rotting fruit.

Despite the obvious differences between flies and humans and the phylogenetic lineage divergence for ~800 million years, basic molecular and cellular processes that control body functions appear conserved across evolution. 14.9 % of human genes were predicted to have orthologues in the fly and, in turn, 46 % of fly genes might be conserved as orthologues in humans (Shih, Hodge and Andrade-Navarro, 2015). Moreover, 75% of all known human disease related genes may be assigned homologues in the fly (Bier, 2005; Reiter *et al.*, 2001). Thus, comparative studies employ the fruit fly as a model in distinct biological disciplines, aiming at insights to benefit human well-being and health.

From the beginning, most of the studies employing *Drosophila* rely on genetically modified animals. The manipulation of gene expression facilitates functional gene analyses to resolve the physiological background of a gene product. Stable genetic manipulation is facilitated by random or targeted genome editing (e.g. chemical mutagenesis, transposable elements, CRISPR)

(Lin, Chang and Chan, 2014), yielding gene knockouts or defined (non-) synonymous mutations, gene knockdowns (RNA interference), ectopic or overexpression. A central tool kit in the fly to manipulate gene expression and certain cell properties are binary and ternary expression systems. These genetic systems facilitate temporally and spatially controlled/targeted expres-



**Figure 1.5 | Schematic of the GAL4/UAS gene expression system in *Drosophila*.** To facilitate the spatially and temporally targeted expression of a gene, GAL4 driver lines are crossed to UAS responder lines to combine the *gal4* and UAS allele in F1 progeny. GAL4 is a transcriptional activator protein, which binds to the UAS to initiate transcription of the target gene. The *gal4* allele carries a gene specific promoter, which defines the site and time of expression of the GAL4 protein. The UAS (upstream activating) sequence is fused to a target/effector gene that eventually mediates the cellular effect. With elements from (Muqit and Feany, 2002).

sion of a gene of interest/in a tissue of interest. Although there are different systems, the next paragraph will only introduce the most famous and simplest, which is the GAL4/UAS system. The GAL4/UAS system is based on a gene expression control system that was derived from yeast (Brand and Perrimon, 1993). The mechanism is based on two distinct genetic elements: (1) a driver construct, composed of a defined promoter region of a gene of interest, which is fused to the gene of the transcriptional activator protein GAL4 and (2) a responder construct, which carries the binding site for GAL4, the upstream activating sequence (UAS), as well as the sequence of a target or effector gene, e.g. reporter gene (see below for further examples). To facilitate the targeted expression of the target/effector gene, driver and responder lines are crossed to combine the allelic elements. F1 progeny possess the combination of driver and effector allele in their genotypes; GAL4 is synthesised and binds to the UAS to initiate target/effector gene transcription (Figure 5).

In neurogenetic manipulations, possible target/effector genes can be fly genes (to achieve ectopic or overexpression, dsRNAi constructs), human genes (e.g. disease-related

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proteins/isoforms) and more general effector genes that affect the cells in a broader way (cell activating/inhibiting/ablating genes). Concerning the latter, a series of sophisticated molecular tools is available with varying potency and inducibility by pharmacological substances, temperature or light (Pauls *et al.*, 2014). Light activated effectors represent the group of optogenetic tools, that combine optical (light absorbing) and genetic elements to enable the precise control of the excitement state of neurons (on/off). In *Drosophila*, a number of different variants has been established. Channelrhodopsin-2 (ChR2) is a microbial rhodopsin, derived from the green algae *Chlamydomonas reinhardtii*. It is a selective cation channel, that, in the presence of its cofactor all-*trans*- retinal and upon blue light stimulation, leads to a depolarisation of the cell and the initiation of action potentials (Nagel *et al.*, 2003; Pulver *et al.*, 2009). Channelrhodopsins in general have been modified to function effectively without its cofactors (ChR2-XXL, Dawydow *et al.*, 2014) or by red light activation to improve stimulation by enhancing tissue penetration (ReaChR; Inagaki *et al.*, 2014; Lin *et al.*, 2013). Another group of optogenetic effector proteins is comprised of light-activated enzymes, such as photoactivated adenylate cyclases (PACs) that generate cAMP upon light exposure. Of these, representatives have been isolated from the flagellate *Euglena gracilis* (euPACs, PAC $\alpha$  and  $\beta$ ; Iseki *et al.*, 2002) and the bacterium *Beggiatoa* (bPAC; Stierl *et al.*, 2011), which are both blue light-activated, or engineered as fusion proteins like the infrared light-activated adenylate cyclase (IlaC; Ryu *et al.*, 2014).

## 1.5 Aims of the study

DA possesses a central role as chemical messenger and especially neurotransmitter in the human brain and body. It functions in a variety of physiological processes, e.g. the regulation of endocrine function, the creation of emotions and motivation, learning and further cognitive functions, as well as motor control. Imbalances in the DA system have been found to underlie severe pleiotropic disease phenotypes with corresponding losses in quality of life of affected persons. Generally, the DA system is well studied in terms of functional relevance. However, less is known about the precise, context-dependent signalling mechanisms involving receptors and intracellular signalling cascades. These mechanisms are of acute relevance as they provide the potential targets in therapies of DA pathologies.

To support the ongoing research in this field, the use of an invertebrate model is expedient. The DA systems of humans and flies share homologous properties on the ultrastructural and molecular level, e.g. receptors and signalling mechanisms, as well as in their functional involvement.

Based on this and the available genetic “tools”, *D. melanogaster* provides an ideal model to further elucidate the complex signalling mechanisms. In this light, I studied different elements of the fly’s DA system. My research focus was on the functional characterisation of the DA receptors DopR and D2R in a physiological context.

The major aims of this theses were:

- (1) to understand the physiological role of dopaminergic signalling in the simple model *Drosophila*,
- (2) to assign specific functions to specific receptor subtypes (focused on DopR and D2R).

## 2 MATERIALS AND METHODS

### 2.1 Materials

#### 2.1.1 Antibodies

**Table 1 | Primary antibodies**

Antibody	Antigen	Source
$\alpha$ -Dh44 (rabbit)	Diuretic hormone 44	Jan A. Veenstra, University of Bordeaux, France
$\alpha$ -NC82 (mouse)	Bruchpilot	Developmental Studies Hybridoma Bank, University of Iowa
$\alpha$ -PDF C7 (mouse)	Pigment dispersing factor	Developmental Studies Hybridoma Bank, University of Iowa
$\alpha$ -GFP 12A6-b (mouse)	Green fluorescent protein	Developmental Studies Hybridoma Bank, University of Iowa
$\alpha$ -GFP (rabbit)	Green fluorescent protein	Sigma-Aldrich

**Table 2 | Secondary antibodies**

Antibody	Source
$\alpha$ -mouse AF 488	Jackson ImmunoResearch
$\alpha$ -rabbit DL 488	Jackson ImmunoResearch
$\alpha$ -rabbit DL549	Jackson ImmunoResearch

#### 2.1.2 Chemicals and reagents

Agar-agar	Roth
Blue food dye	Ruth
Brewer's yeast	Leiber
Calcium chloride dihydrate ( $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$ )	Roth
Coconut fat	Peter Kölln KGaA
Corn meal	Davert GmbH
Corn sirup (powder)	Raab Vitalfood

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D(-)-arabinose ( $\geq 98\%$ )	Sigma-Aldrich
Dopamine hydrochloride	Sigma-Aldrich
D(-)-Fructose	Sigma-Aldrich
D-(+)-Glucose monohydrate	Roth
D(+)-Trehalose dehydrate	Roth
HEPES	Roth
Magnesium chloride hexahydrate ( $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$ )	Roth
Molasses	Biohof Heide Licht
<i>my</i> -Budget low-melt agarose	Bio Budget Technologies GmbH
Nipagin	Roth
Normal goat serum	Sigma-Aldrich
Paraformaldehyde powder EM grade	Polysciences Inc.
Potassium chloride (KCl)	Merck
Propionic acid	Roth
RapiClear® 1.47	SunJin Lab Co.
Schneider's <i>Drosophila</i> Medium	Genaxxon Bioscience
Silica gel	Roth
Soda lime	Roth
Sodium chloride (NaCl)	Roth
Sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4$ )	Appli Chem
Sodium hydrogen carbonate ( $\text{NaHCO}_3$ )	Merck
D-(+)-Sucrose	Diadem
Sugar beet syrup	Kanne Brottrunk GmbH
Triton X-100	Roth
Tween-20	Roth

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Triglycerides	Thermo Scientific
Triolein	Sigma-Aldrich
Yellow food dye	Ruth

#### 2.1.4 Buffers and solutions

IHC wash buffer: 0,3 % (v/v) Triton X-100 in 1 x PBS

IHC blocking buffer: 5 % (w/v) goat serum in wash buffer, stored at -20°C

Hemolymph-like saline (HL3.1): 70 mM NaCl, 5 mM KCl, 20 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 1.5 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 10 mM NaHCO<sub>3</sub>, 115 mM sucrose, 5 mM trehalose, 5 mM HEPES in deionized water (pH7.1), filter sterilised and stored at 4°C.

10 % Nipagin: 10 % (w/v) Nipagin in ethanol

10 % Propionic acid: 10 % (v/v) Propionic acid in deionised water

2.1.5 2 % Paraformaldehyde (PFA): 2 % (w/v) PFA in 100 ml 1x PBS (pH 7,4), stored at -20°C

Phosphate buffered saline (PBS): 58.33 mM NaCl, 74.55 mM KCl, 141.96 mM KH<sub>2</sub>PO<sub>4</sub>, 136.09 mM Na<sub>2</sub>HPO<sub>4</sub> in deionised water (pH 7.4), filter sterilised

#### 2.1.5 Fly food

Standard medium: 31.25 g corn meal, 31.25 g brewer's yeast, 5 g agar- agar, 10 g glucose, 15 g molasses, 15 g sugar beet syrup in 500 ml deionised water. Boiled up before autoclaving. After cooling to 60°C, 15 ml 10 % nipagin and 5 ml 10 % propionic acid were added.

Monitor medium (used in locomotor activity assays): 1g agar, prepared in 25 ml deionised water. After cooling to 60°C, 2.5g glucose dissolved in 25 ml deionised water, as well as 1.5 ml nipagin were added.

High fat medium: 150 mg agar-agar, 200 mg yeast, 700 mg corn sirup (powder), 1 g cocoa butter in 10 ml deionised water. After cooling to 60°C, 0.3 ml 10% nipagin were added.

## 2.1.6 Fly strains

Table 3 | Fly strains, genotype and source

Name	Genotype description	Source
D2R Δ2	D2R Δ2;+;+ Includes parallel knockout of the gene CG17003, of which the function is unknown.	(Scholz-Kornehl, 2015; Scholz-Kornehl and Schwärzel, 2016)
dumb <sup>2</sup>	PBac{WH}Dop1R1 <sup>f02676</sup>	Bloomington <i>Drosophila</i> Stock Center
WT (w <sup>1118</sup> ) w <sup>1118</sup> (in crosses)	w <sup>1118</sup>	Bloomington <i>Drosophila</i> Stock Center
WT (CS)		Bloomington <i>Drosophila</i> Stock Center
UAS-bPAC	w*;P{UAS-bPac.S}/CyO	Martin Schwärzel (FU Berlin)
UAS-D2R-dsRNA		Martin Schwärzel (Draper <i>et al.</i> , 2007)
20xUAS-6xGFP	y1 w*; wgSp-1/CyO, P{Wee-P.ph0}BaccWee-P20; P{20XUAS-6XGFP}attP2	Bloomington <i>Drosophila</i> Stock Center
UAS-CG17003-dsRNA	KK	VDRC (ID 101273)
UAS-Epac camps 50 A	w <sup>1118</sup> ; P{UAS-Epac1-camps}50A	Bloomington <i>Drosophila</i> Stock Center
Dh44-Gal4	w <sup>1118</sup> ; P{Dh44-GAL4.TH}2M	Bloomington <i>Drosophila</i> Stock Center
D2R-Gal4		Best Gene Inc (El-Kholy, 2010)
DopR-Gal4		Best Gene Inc (El-Kholy, 2010)
PDF-Gal4		
Th-Gal4	w*; P{ple-GAL4.F}3	Bloomington <i>Drosophila</i> Stock Center
tubP-Gal4	y1 w*; P{tubP-GAL4}LL7/TM3, Sb1 Ser1	Bloomington <i>Drosophila</i> Stock Center

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### 2.1.7 Equipment

Thermometer and humidity detector	TFA Dostmann Wertheim
Omni Bead Ruptor 24	Omni International
Power bar V2 LED strip Osram Oslon royal blue	Lumitronix
Thermomixer comfort	Eppendorf
Centrifuge 54515 D and 5417 R	Eppendorf
Micropate reader LEDETECT 96	Delux Labortechnik GmbH
DAM2	TriKinetics, Waltham
LAM	TriKinetics, Waltham
PSIU9 power supply interface unit	TriKinetics, Waltham
DFM and MCU (FLIC)	Pletcher Lab, University of Michigan
Fluorescence microscope Axio Imager Z1	Zeiss
Axioskop2 FS plus	Zeiss
ApoTome	Zeiss
Axiocam MRm camera	Zeiss
Leica S6E	Leica

### 2.1.8 Additional materials

24- and 96-well plate	Sarstedt
Adhäsive luftdurchlässige Verschlussfolie	Nerbe plus
Tissue culture flask	Sarstedt
Petri dish (55 mm)	Nerbe plus
Fly vials (PS, 50 ml)	Nerbe plus
Fly vial plugs (cellulose, 30 mm)	Nerbe plus
Parafilm	

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Capillaries (d = 1.15 mm)	BRAND GmbH + CO KG
Plasticine	Pelikan
Screw-cap tube (2 ml)	Sartstedt
1.5 ml tube	Nerbe plus
Ceramic beads (2.6mm-2.8mm)	Biolabproducts GmbH
Cover slips (20 x 20 mm, 24 x 20 mm)	Roth
Object slides	Roth

### 2.1.9 Software

Microsoft office Excel and Powerpoint 2010

The Ultimate Reader of Dung (T.U.R.D.) (Wayland *et al.*, 2014)

ImageJ-win64 and ActogramJ plugin (Schmid, Helfrich-Förster and Yoshii, 2011)

AxioVisionSE64 Rel.4.9

GraphPad Prism 4.0 and 7.0

Biology Bundle (Motifolio Inc.)

## 2.2 Methods

### 2.2.1 Fly culture and crosses

*Drosophila* stocks were kept at 21°C and 16°C on standard medium. Flies to be employed in experiments were reared and kept at 25°C on standard medium unless otherwise stated.

UAS responder line virgin females were crossed Gal4-driver males. For control genotypes, driver and responder lines were crossed to wild type flies (WT ( $w^{1118}$ )) respectively. In case of crosses involving the fly line *UAS-bPAC*, the crosses were kept in constant darkness. F1 progeny were used in experiments.

The flies were aged for 3-5 days after eclosure at 25°C (30°C in case of RNAi crosses) before employment in experiments.

Anaesthetisation was carried out on ice and with nitrogen gas.

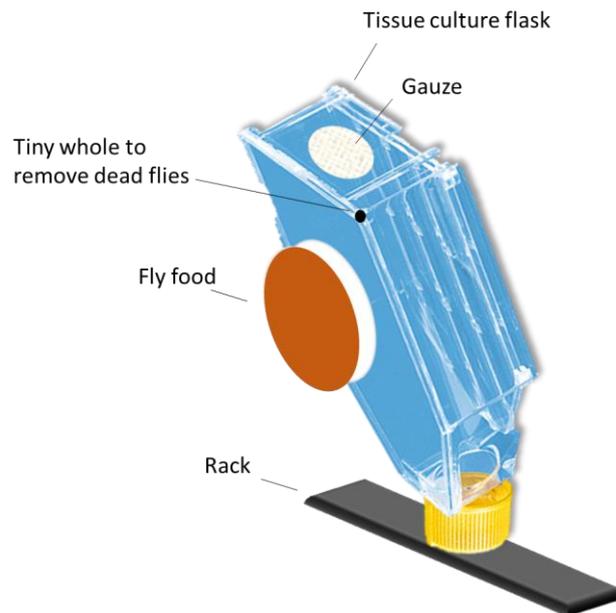
## 2.2.2 Survival assays

### 2.2.2.1 General life span assay

Flies were allowed to eclose for 1-3 days before assay start. For the assay, males and females were separated and placed into purpose adapted tissue culture flasks (Figure 2.1) in cohorts of 25 -50 individuals. The flies were kept on standard medium at 25°C/20°C light/dark cycle (12hrs:12hrs). Dead flies were counted and removed every second day. The food was changed every second to fourth day.

In case of crosses involving RNAi, the assay was conducted at 30°C constant temperature. In case of crosses involving the fly line *UAS-bPAC*, the flies were exposed to blue light (~240 lx) in a 12hrs:12hrs light/dark and 25°C/20°C cycle.

Log-rank test was generally used for statistical evaluation. The maximum life span was determined from the mean life span ( $\pm$  sd) of the oldest 10% cohort of individuals.



**Figure 2.1 | Life span assay.** Tissue culture flasks were purpose adapted. The flasks were placed into a rack in bottom-up orientation.

### 2.2.2.2 Starvation resistance assay

Flies were collected within 1-3 days after eclosure and aged for 6-8 days on standard medium at 25°C before assay start. The assay was conducted in normal fly vials prepared with 5 ml 2 % agar bottom and a filter paper strip that was soaked with 600  $\mu$ l deionised water. The filter paper

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and water was renewed daily. Dead flies were counted every 3 hours from 08:00 am to 20:00 pm.

### 2.2.2.3 Desiccation tolerance assay

Flies were collected within 1-3 days after eclosure and aged for 6-8 days on standard medium at 25°C and constant darkness before assay start. For the assay, flies were placed individually in wells of a 24-well-plate, which was covered with an air-permeable sealing film. The well plates were arranged in a plastic box. In addition, the box was equipped with 2 x 200 g of silica gel to reduce humidity and a digital thermometer and humidity detector. Because of the blue light application the temperature increased to 26°C, humidity dropped to 20 % and remained constant. The plastic box was covered, sealed with Parafilm, and placed into a blue light box. The assays were started in the late evening and counting of dead flies was started the following morning (30 min intervals). Blue light was constantly applied (240 lx). Individuals were counted as dead, when they were lying on the side/back and did not show any movements after slight mechanical stimulation.

## 2.2.3 Metabolic/Physiology assays

### 2.2.3.1 Determination of metabolic rates

The metabolic rate is determined by the assessment of CO<sub>2</sub> production as a measure for the breathing rate (O<sub>2</sub> consumption = metabolic rate). The assay was described by Yatsenko *et al.* (2014).

Briefly, a capillary micropipette was fixed at the opening of a 1000 µl pipette tip. 3 pieces of water-soaked soda lime were enclosed by 2 pieces of foam (one at the bottom and one at the top) and pushed to the bottom of the pipette tip. 3 flies were transferred to the “fly chamber” of the respirometer, which was subsequently sealed with plasticine. The respirometer was carefully placed in a polystyrene rack and subsequently placed into a respirometer chamber, which contained coloured water. The capillary submerged into the water (Figure 2.2). Treatment and control flies were always run in one chamber; in addition, 1-2 blank respirometers were included per run. The chamber was covered with a glass plate to maintain constant conditions within the chamber and the flies were allowed to adapt for 15 min. A photograph was taken at the beginning and end of the assay (after 1 or 2 hours) to determine the difference of the fluid level in capillaries. In case of crosses involving the fly line *UAS-bPAC*, the flies were exposed to blue light (~240 lx) over the course of the experiment.

The amount of CO<sub>2</sub> production (μl per fly and hour) was calculated with the following formula:

$$\mu\text{l} \frac{\text{CO}_2}{\text{hr} * \text{fly}} = \frac{(\pi \times R^2) \times (\Delta d - \Delta c) \times 1000}{n \times \text{hr}}$$

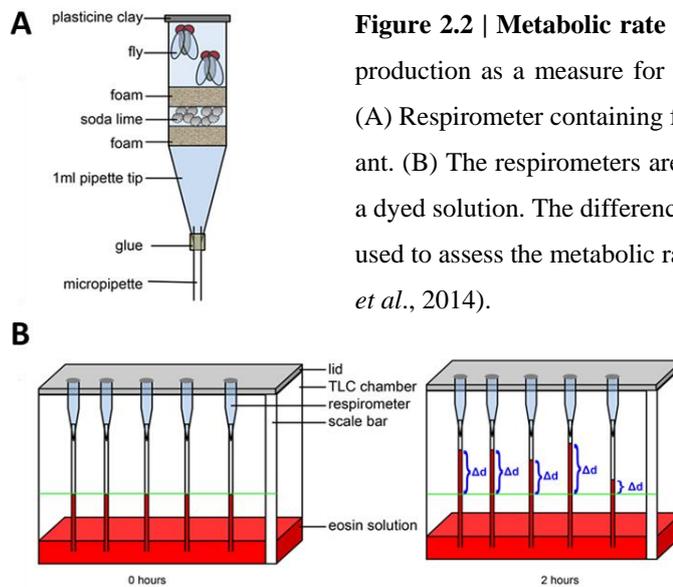
R: radius of capillary micropipette (cm)

Δd: height of the liquid in experimental samples far from the colored solution (cm)

Δc: height of the liquid in negative controls far from the colored solution (cm)

n: number of flies used

hr: hours of assay performance



**Figure 2.2 | Metabolic rate assay.** The assay determines CO<sub>2</sub> production as a measure for O<sub>2</sub> consumption (metabolic rate). (A) Respirometer containing flies and soda lime as CO<sub>2</sub> absorbant. (B) The respirometers are placed into chambers containing a dyed solution. The difference in fluid level in the capillaries is used to assess the metabolic rate. Scheme taken from (Yatsenko *et al.*, 2014).

### 2.2.3.2 Coupled colorimetric assay

The body fat content of flies was assessed by the determination of the total triacylglyceride (TAG) level after (Hildebrandt, Bickmeyer and Kühnlein, 2011).

Before the assay, flies were kept on high fat diet for one week to enhance potential effects. 5 female flies or 8 males were used per assay replicate. The flies were removed from the food, placed into a 2 ml screw-cap tube, weighted and subsequently homogenised in 1 ml of 0.05 % Tween-20. Homogenisation was carried out by the aid of 3 ceramic beads and a bead ruptor at a speed of 3.25 m/s for 2 min. The homogenates were subsequently incubated at 70°C for 5 min, followed by 10 min centrifugation at 3000 x g to collect the debris at the bottom of the tube. 500 μl of the supernatant were removed and stored at -20°C until measurement.

A standard curve was established with a 6-step dilution series of glycerol trioleate (range: 100 μg/50 μl – 3.12 μg/50 μl). Tween-20 blank was included. 50 μl of fly homogenate (prewarmed to 37°C, in triplicates) or standard dilution (in duplicates) were transferred into the wells of a

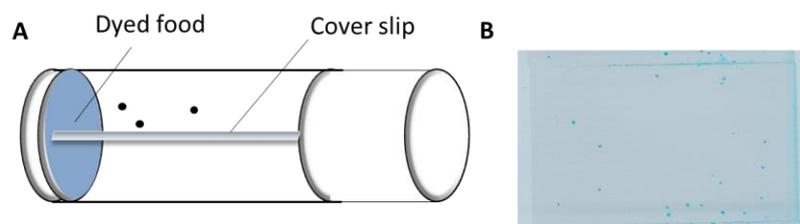
96-well microtiter plate and the absorbance was determined at 562 nm ( $T_0$ ). Afterwards, 200  $\mu$ l of Thermo Infinity triglyceride solution (prewarmed to 37°C) was added to the wells and the plate was incubated for 30 min. at 37°C and 120 rpm. Again, absorbance was determined at 562 nm ( $T_1$ ). Individual absorbances were blank corrected and the amount of TAG was determined based on the standard curve.

### 2.2.3.3 Excretion assay

The excretory phenotype of flies was determined to assess the intestinal integrity as well as to obtain information on the level of diuresis. As the Malpighian tubules secrete fluid into the intestine at the midgut-hindgut boundary, the water content of faecal spots facilitates the assessment of diuresis rate (Cognigni, Bailey and Miguel-Aliaga, 2011). The excretory phenotype was assessed at the basis of graphical characteristics of excreta (Wayland *et al.*, 2014).

Flies were kept on blue dyed standard medium (0.5% dye) one day before the assay start. Fly food vials were prepared with blue dyed food and a cover slip (24 x 20 mm), which was placed into the vial in a way that flies got trapped on one side (Figure 2.3). 3 flies were placed into the vial. After 24 hours, the cover slip was removed and scanned (600 dpi) for further graphical analyses. Thus, the data reported below refers to the portion of faecal spots on the cover slip.

The graphical parameters of excreta were determined by employing a software tool (The Ultimate Reader of Dung, T. U. R. D., Wayland *et al.*, 2014). In this study, information on the number of spots (excretion rate), the area and lightness values were used. The number and size of faecal spots indicates excreted amount; lightness values are correlated with colour intensity and thus water content of excreta. In case of crosses involving the fly line *UAS-bPAC*, the flies were exposed to blue light (~240 lx) over the course of the experiment.



**Figure 2.3 | Excretion assay.** (A) Fly vials were prepared with blue dyed food and a cover slip. (B) Scan of a cover slip that was used to obtain information on the graphical characteristics of excreta (Wayland *et al.*, 2014).

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#### 2.2.3.4 Determination of intestinal transit times

For the determination of intestinal transit times, female flies were starved overnight. 24-well plates were prepared by placing a drop of dyed yeast paste into the individual wells. Flies were distributed among the wells and the wells were examined for faecal spots every 15 min over the course of 3 hours. In case of crosses involving the fly line *UAS-bPAC*, the flies were exposed to blue light (~240 lx) over the course of the experiment.

#### 2.2.3.5 Determination of food intake by using the FLIC

The amount of food intake was assessed by employing the Fly Liquid-Food Interaction Counter (FLIC) (Ro, Harvanek and Pletcher, 2014). The FLIC principle is based on the monitoring of a simple electronic circuit that is created by a fly in phases of contact with liquid food, which can be correlated with feeding or tasting events. The FLIC system consists of 3 components. (1) The *Drosophila* Feeding Monitor (DFM) consists of a set of 12 feeding wells, each equipped with a signal detection circuit (detects feeding/tasting events). (2) The Master Control Unit (MCU) coordinates the data in case of multiple attached FLICs, provides first data processing and forwards the data to the PC monitoring software, which represents the third component (3). In this study, 5 % glucose (in deionised water) was used as liquid food. Feeding events (counted in units with values >100) of males were determined over the course of 24 hours. The data was analysed with Excel.

### 2.2.4 Behavioural assays

#### 2.2.4.1 Two-choice assay

The two-choice assay was based on the study by Dus *et al.* (2015). In principle, groups of 10 flies were given the choice between two sugars, one nutritive and one non-nutritive sweeter sugar. The sugars were colour coded to facilitate visibility after ingestion. Based on the colour visible through the abdomen, the sort of ingested sugar was concluded. The flies were starved for 18 hours prior to the assay. The sugar media were prepared with 1.5 % low-melt agarose as base and filled into 1.5 ml tube caps. D-fructose represented the nutritive sugar and was used at 25 mM concentration. D-arabinose represented the non-nutritive sugar and was used at 80 mM concentration. The arabinose sugar medium was first stained with blue dye (0.5% final concentration) and the fructose medium was stained with yellow dye (1% final concentration). However, the assignment of colours was interchanged, so that both colour/sugar combinations

were considered in the assay to avoid colour-preference bias. Two prepared tube caps of the same sugar/colour combination were placed into petri dishes (Ø 55 mm) as well as the flies (in groups of ten, males and females separated). The petri dishes were then placed into an LED-box to facilitate blue light exposure (240 lx). After 2 hours, the colours visible in the abdomen were scored. The preference index was calculated with the following formula (Dus *et al.*, 2015):

$$PI (\%) = \frac{((\text{no. flies that ate food A} + 0.5 * \text{flies that ate both}) - (\text{no. flies that ate food B} + 0.5 * \text{flies that ate both}))}{\text{no. total flies that ate}}$$

#### 2.2.4.2 Recording of locomotor activity

Locomotor activity data have been generated by simple activity recordings employing the *Drosophila* Activity Monitoring system (TriKinetics). The monitor records how often a fly crosses an infrared beam that penetrates the monitor vial at medium height. The individual activity counts of 3-5 day old flies on glucose-agar food were monitored over 3 days under a 12h:12h light-dark (LD) cycle at 25°C (day)/20°C (night) by the use of the DAM 2 (*Drosophila* Activity Monitor). The data obtained from 3 days has been pooled per individual for analysis. The data was summed into 5 min bins by using the DAMFileScan software.

##### 2.2.4.2.1 Total activity and activity level

The total activity represents the average of activity counts across all data points.

The total activities over day periods represent the sum of activity counts per specified time interval. The “Morning” phase corresponds to Zeitgeber time 6 – 12; the “Afternoon” phase corresponds to Zeitgeber time 12 – 18; the “Evening” phase corresponds to Zeitgeber time 18 – 24; the “Night” phase corresponds to Zeitgeber time 0 – 6.

To determine the activity level (activity in waking minutes) the activity data was corrected for sleep phases. The activity level represents the average of activity counts in wake phases (Andretic and Shaw, 2005).

##### 2.2.4.2.2 Sleep analysis

A 5 min bin of inactivity was considered as sleep unit. Consecutive sleep units without interruption were considered as sleep bout. In order to determine the number of sleep bouts, the data was arranged in an order that a day was started at Zeitgeber time 0. The number of sleep bouts

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was determined per day, for the light phase (Zeitgeber time 0 – 12) and night phase (Zeitgeber time 12 – 24). Sleep latency represents the minutes of sleep until the first sleep bout in the night phase (Andretic and Shaw, 2005).

#### 2.2.4.2.3 Circadian rhythm analysis

Physiological processes and behaviour are coordinated in patterns throughout the day by inner circadian timing systems/circadian clocks. This rhythmic control of body function is highly conserved and present from bacteria to men. In the *Drosophila* model, locomotor activity is conveniently accessible and thus the behaviour of choice to study the circadian clock system (Chiu *et al.* 2010). Moreover, locomotor behaviour can be employed to study general movement abilities and associated disorders.

To record activity for circadian rhythm analysis, the LAM 25 (Locomotor Activity Monitor) was used. Flies were assayed individually. They were entrained for 5 days under for a 12h:12h light-dark (LD) cycle at 25°C (day)/20°C (night) and subsequently released into a free-running period of 7 days in constant darkness at 20°C. Actogram J was used to analyse circadian rhythm data. The actograms represent average actograms (smoothed by convolution with a Gaussian kernel). The free-running period was determined by using the Chi-square method. The result was verified manually.

### 2.2.5 Microscopic analyses techniques

#### 2.2.5.1 Immunohistochemistry staining

Fly brains and VNC were dissected in ice cold Schneider's Insect medium and fixed for 55 min in 2 % PFA at room temperature. The brains were subsequently washed in PBT (4 x 10 min) and blocked with 5% NGS for 1.5 hours at room temperature. After removal of the blocking buffer, the primary antibody was applied and stored at 4 °C overnight. The antibody was washed off in 3 washing steps with PBT (30 min each), which was followed by incubation with the secondary antibody (overnight at 4°C). Again, the antibody was removed in 3 x 30 min washing steps with PBT. The brains were mounted on object slides and cleared with RapiClear. Images were taken with an Axio Imager Z1 fluorescence microscope with ApoTome (Zeiss). Final concentrations used for primary antibodies:  $\alpha$ -Dh44 (rabbit) 1: 200,  $\alpha$ -NC82 (mouse) 1:50,  $\alpha$ -PDF (mouse) 1:15,  $\alpha$ -GFP (mouse) 1:300,  $\alpha$ -GFP (rabbit) 1:300. Secondary antibodies were used in 1:300 concentrations.

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### 2.2.5.2 Quantification of Dh44 fluorescence intensities

To test for Dh44 release from PI cells, Dh44 fluorescence intensities were quantified in two approaches.

- (1) DA application. Wild type ( $w^{1118}$ ) brains were dissected and subsequently incubated with a solution containing 1 mM DA (in HL3.1) or control buffer (HL3.1) for 30 min. This was followed by fixation in PFA and the standard staining protocol was continued. Dh44 antibody was used in 1:800 final concentration.
- (2) bPAC application. Brains of Dh44>bPAC flies were dissected under red light conditions followed by blue light application for 30 min or incubation in darkness (control). This was followed by the standard staining protocol. Dh44 antibody was used in 1:800 final concentration.

Images were taken with an Axio Imager Z1 fluorescence microscope with ApoTome (Zeiss).

The fluorescence intensities were determined with Image J based on the following formula:

Fluorescence intensity = Integrated Density – (Area of region of interest \* mean fluorescence of background).

### 2.2.5.2 Live cell imaging (cAMP imaging)

For cAMP imaging *Dh44>UAS-Epac camps 50 A* flies were used. UAS-Epac camps represents a cAMP adaptor protein that possesses two fluorescent dyes, CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein). In a cAMP unbound state fluorescence resonance energy transfer (FRET) occurs between these two fluorescence proteins. However, when the binding of cAMP leads to a conformational change, which is accompanied by a decrease in FRET signal (Ponsioen *et al.*, 2004).

Fly brains were dissected and placed into a small self-made chamber. The brains were allowed to adapt for 20 min before recording was started. In the recording series of CFP/YFP fluorescence intensities, at first control buffer (HL3.1) was applied, which was followed by DA (1 mM) application to the same brain. The recording was performed with an Axioskop2 FS plus (Zeiss) for 30 min, the solutions were applied in minute 5 after the start of the recording. The data was obtained from one region of interest per brain, relating to one Dh44 PI cell.

### 2.2.6 Statistical analysis

Statistical evaluation of data was performed with GraphPad Prism 4.0 and 7.0. Generally, the data distribution was tested for normality by using Shapiro-Wilk normality test. If 2 groups were compared and the data fit the criteria, unpaired student's t test was used. In cases of non-normally distributed data, the Mann Whitney test was applied. In cases of 3 groups comparison, one- or two way ANOVA was performed and followed by Bonferroni or Tukey's multiple comparison test. p values were defined as follows: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.0001$ .

## 3 RESULTS

### 3.1 Role of DopR in metabolism and activity regulation

#### 3.1.1 *dopr* promoter activation pattern in the brain and VNC

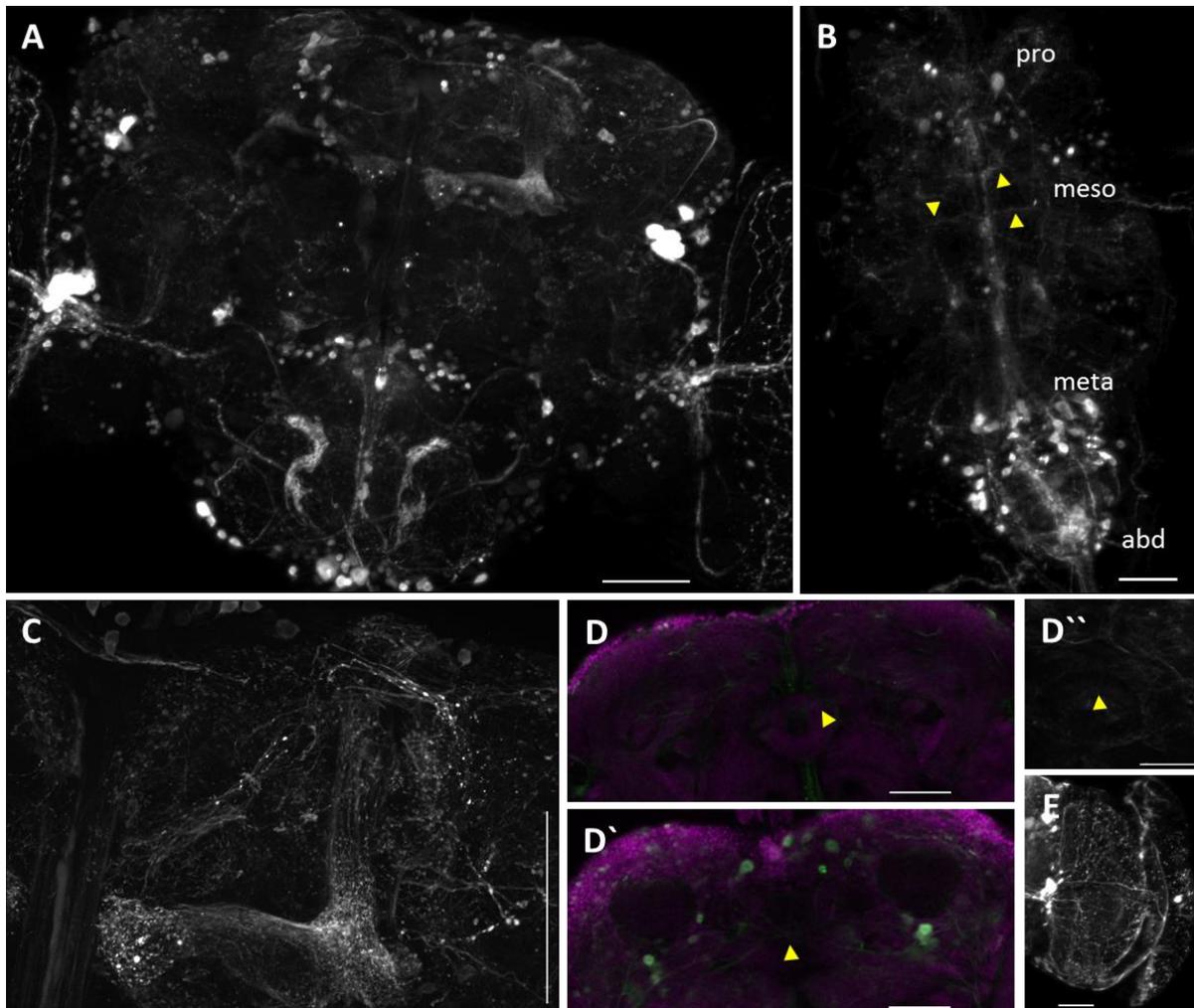
The *dopr-Gal4* fly line has been generated in a previous study and has moreover been employed to characterise the receptor promoter activation pattern based on GFP reporters (El-Kholy, 2010; Stephano, 2013). However, we again used this driver line to examine the pattern of the enhanced GFP reporter *20xUAS-6xgfp*.

The expression of a soluble GFP in the pattern of *dopr* promoter activity revealed an extensive cellular network of *dopr* gene activation in the CNS of the fly with distinct clusters of cell bodies located in different brain regions and processes extending throughout the brain hemispheres (Figure 3.1). In general, although the signal in regional substructures appeared variable among individual brains, the overall DopR expression pattern described in previous studies was reproduced (El-Kholy, 2010; Kim *et al.*, 2003; Stephano, 2013).

Within the adult central brain a strong labelling of processes was seen in the MB  $\alpha/\beta$  and  $\alpha'/\beta'$  lobes, including the heel. Moreover, signal was detected in the cell bodies of the calyx although labelling of the whole substructure was incomplete (Figure 3.1 A and C). The CC did not show prominent *dopr* promoter activation, which is in contrast to what was reported from direct receptor IR (Kim *et al.*, 2003). However, GFP positive processes were detected in association with the EB (Figure 3.1 D) and weak expression was present in the FSB (Figure 3.1 D''). Posterior to the FSB, ventral to the PB (Stephano, 2013), a bilateral cluster of strongly labelled cell bodies existed from which processes extended towards the midline to intersect and further project to dorsal brain regions (Figure 3.1 D``).

Additional DopR producing cell body clusters were detected in neurosecretory cells of the dorsal midbrain (*pars intercerebralis*) with projections along the midline, associated with the ventral region of the AL and within the GNG, which is additionally permeated by a broad network of labelled neurons. Within the GNG, cell bodies were located ventrally and strong GFP signal surrounded the oesophagus foramen (Figure 3.1 A). Very strong signal is also seen within the elements of the circadian clock system and large ventral lateral neurons (ILN<sub>vs</sub>) stand out as most prominent. Furthermore, the OL possessed a broad meshwork in all substructures, including LO, ME and L (Figure 3.1 E).

Neurons along the cervical connective (not shown) extended to the abdominal ganglion (abd) within the VNC (Figure 3.1 B). Cell bodies of the VNC were located in the pro- and metathorax neuromeres (pro, meta) and the abd. The mesothorax neuromere (meso) possessed GFP positive commissures. Scale bars = 50  $\mu$ m.

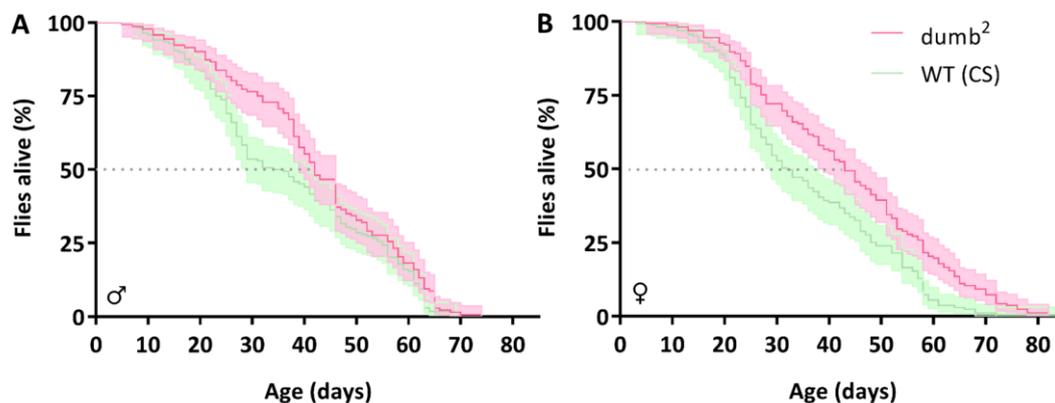


**Figure 3.1 | *dopr* promoter activation pattern in the adult CNS.** Immunohistochemical analysis of GFP reporter signal (white and green) in *dopr-Gal4 > 20xUAS-6xgfp* flies.  $\alpha$ -NC82 costaining of neuropil regions is shown in magenta. GFP signal associated with *dopr* promoter activation was detected in cell bodies and neuronal processes throughout different regions of the brain and VNC. (A) Overview of *dopr* expression in the central brain. Most prominently labelled substructures are the MB lobes (C), ramifications surrounding the oesophagus foramen and extended meshwork covering the GNG, cell bodies within the ventral GNG, the ILNvs and OL (E). Further signal was detected associated with the CC, especially the EB (D, single optical section of an image stack); arrow head indicates labelled neurons (green) associated with the EB (magenta). More intensely labelled neurons were located posterior to the EB of which processes crossed at the midline (arrow head) and further extended to dorsal brain regions (D''). The FSB appeared only weakly labelled (D'', arrow head). Within the VNC labelled cell bodies are found in the pro- and metathorax neuromere (pro, meta) as well as the abdominal ganglion (abd, B). Additionally, commissures (arrow heads) were labelled in the mesothorax neuromere (meso). Scale bars = 50  $\mu$ m.

### 3.1.2 DopR function is associated with metabolic traits

#### 3.1.2.1 DopR deficiency increases survival probabilities

To assess the overall physiological relevance of DopR, we analysed the survival times of *DopR*-deficient flies (*dumb<sup>2</sup>*). Both, male and female mutants showed increased median life expectancies, when compared to their matching controls (WT (CS)). *dumb<sup>2</sup>* males showed an increase in median survival by 20 % (Figure 3.2 A, *dumb<sup>2</sup>* males, median lifespan 42 d,  $n = 5$ , total 142 flies; WT (CS) males, median lifespan 35 d,  $n = 5$ ; 168 flies; log-rank test \*  $p = 0.0284$ ), whereas in females the effect appeared even stronger with 34 % increase in median survival times (Figure 3.2 B, *dumb<sup>2</sup>* females, median lifespan 43 d,  $n = 5$ , total 165 flies; WT (CS) females, median lifespan 32 d,  $n = 5$ ; 163 flies; log-rank test \*\*\*  $p < 0.0001$ ).

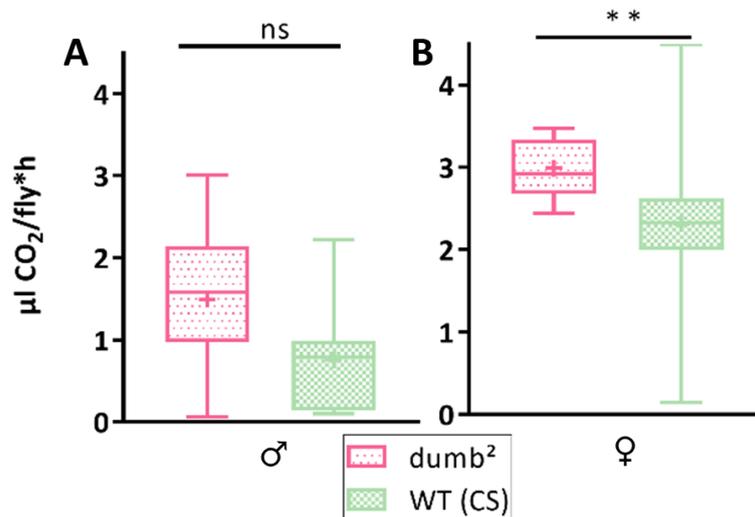


**Figure 3.2 | *dumb<sup>2</sup>* mutants show increased median survival times.** The lifespan assay was performed at 25°C/20°C light/dark cycle (12hrs:12hrs) on standard medium. The proportion of alive flies is plotted in % over time. (A) *dumb<sup>2</sup>* males showed an increase in median lifespan (log-rank test \*  $p = 0.0284$ ; *dumb<sup>2</sup>*: median survival 42 d,  $n = 5$ , 142 flies in total; WT (CS): median survival 35 d,  $n = 5$ , 168 flies in total), as well as *dumb<sup>2</sup>* females (B) (log-rank test \*\*\*  $p < 0.0001$ ; *dumb<sup>2</sup>*: median survival 43 d,  $n = 5$ , 165 flies in total; WT (CS): median survival 32 d,  $n = 5$ , 163 flies in total). Error bands encompass 95 % CI. Dotted lines indicate median survival times.

#### 3.1.2.2 DopR deficiency leads to elevated metabolic activity in female flies

Further insight on the metabolic and health state of the DopR deficient flies was obtained from the determination of metabolic rates by respirometry. In this assay, CO<sub>2</sub> production is analysed as a measure for O<sub>2</sub> consumption; the breathing rate is correlated to the level of metabolism (Yatsenko *et al.*, 2014). As illustrated in Figure 3.3 A, *dumb<sup>2</sup>* flies showed elevated levels of CO<sub>2</sub> production (*dumb<sup>2</sup>* mean CO<sub>2</sub> per fly and hour  $1.493 \pm 0.9077$  (sd)  $\mu\text{l}$ ,  $n = 11$ ; WT (CS) mean CO<sub>2</sub> per fly and hour  $0.7558 \pm 0.7455$  (sd)  $\mu\text{l}$ ,  $n = 7$ ; unpaired t test  $p = 0.0918$ ) although

this only appeared significant for female mutants with an average increase of 29 % when compared to control flies (Figure 3.3 B; *dumb<sup>2</sup> females*  $n = 10$ ; *WT (CS) females*  $n = 12$ ; Mann Whitney test \*\*  $p = 0.0041$ ).



**Figure 3.3 | CO<sub>2</sub> production is elevated in DopR mutant flies.** Metabolic rates of DopR knockout flies were assessed by respirometric determination of CO<sub>2</sub> production per fly and hour. (A) *dumb<sup>2</sup>* males showed a non-significant increase in their metabolic rate level (unpaired t test  $p = 0.0918$ , *dumb<sup>2</sup>*  $n = 11$ , WT (CS)  $n = 7$ ). (B) *dumb<sup>2</sup>* females produced significantly more CO<sub>2</sub> per hour than WT (CS) females, indicating an increased metabolic rate (Mann Whitney test \*\*  $p = 0.0041$ , *dumb<sup>2</sup>*  $n = 10$ , WT (CS)  $n = 12$ ). Plots illustrate the 25% to 75% percentiles (box), median (horizontal line), mean (plus) and min and max values (whiskers). \*\*  $p < 0.01$ .

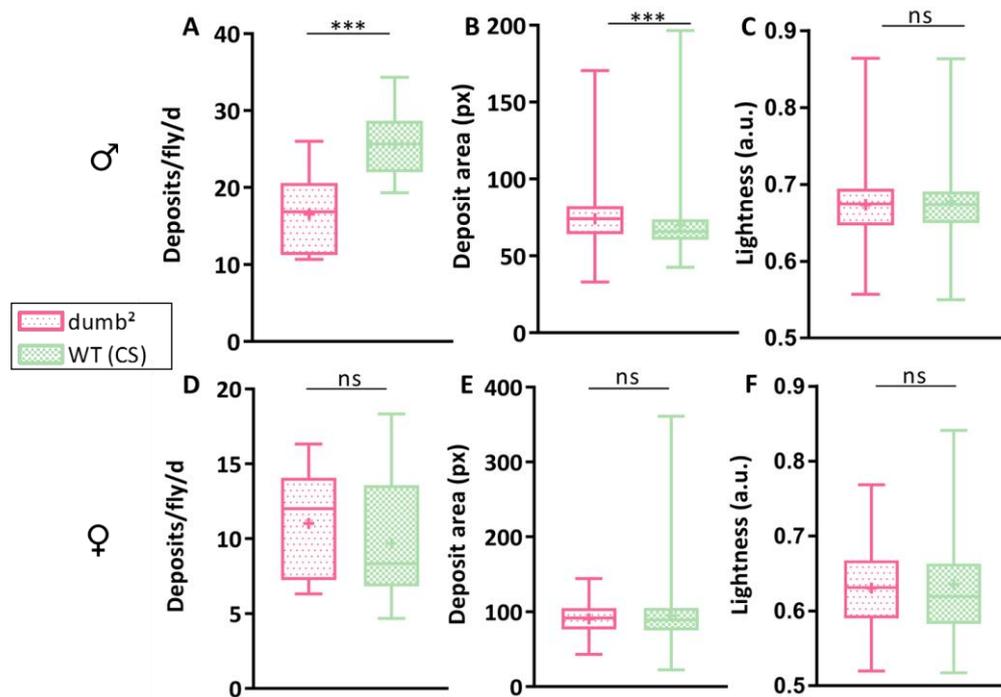
### 3.1.2.3 DopR is associated with faecal output changes in males

Previous studies employing the DopR-Gal4 line and a GFP reporter have found cell-and regional-specific DopR expression in enteroendocrine cells of the larval midgut (Stephano, 2013). However, the function of the receptor in intestinal cells has not been examined so far. To obtain a first insight into the potential role of DopR in intestinal physiology, we analysed the number and graphical characteristics of faecal spots of DopR mutants as described by Wayland *et al.* (2014). For the assay, fly food vials were prepared with blue-dyed standard medium. A cover slip was placed into the vial onto which flies excreted and which was later scanned for a detailed analysis of the deposits.

Male DopR mutants were found to suffer a reduced excretion rate (unpaired t test \*\*  $p = 0.0006$ ,  $n = 10$  each; Figure 3.4 A) but the average deposit size was increased (Mann Whitney test \*\*  $p = 0.0017$ ; Figure 3.4 B). This indicates the excretion of bigger volumes per defecation and is additionally supported by a higher level of integrated optical density (IOD), as from the IOD an increased amount in faecal mass per spot might be inferred (Appendix Figure 1). Moreover,

slight differences in the shape of excreta were found as the spots of mutants appeared less circular (Appendix Figure 1). However, no indication of altered water content of excreta could be detected (Mann Whitney test  $p = 0.8221$ ; Figure 3.4 C).

The excretion phenotype of female DopR mutants was normal as none of the tested parameters was found to be significant ( $n = 10$  each; Figure 3.4 D-F, Appendix Figure 1 C and D).



**Figure 3.4 | DopR is associated with altered excretion characteristics in males.** To assess the excretory phenotype of DopR mutants, groups of three flies were kept in vials with dyed food and cover slips onto which flies deposited excreta. After 24 hours, the cover slips were analysed by employing a software tool to extract the graphical information of excreta (Wayland *et al.*, 2014) Male DopR mutants were found with significantly lower excretion rates (unpaired t test \*\*\*  $p = 0.0006$ ,  $n = 10$  each, A), however, excreta volumes of individual spots were increased (Mann Whitney test \*\*  $p = 0.0017$ , B). The water content of excreta was not different from control flies (Mann Whitney test  $p = 0.8221$ , C). Female mutants were not affected in excretion rate, excreta volume or water (dye) content (D – F). Plots illustrate the 25% to 75% percentiles (box), median (horizontal line), mean (plus) and min and max values (whiskers). \*\*  $p < 0.01$ , \*\*\*  $p < 0.0001$ .

### 3.1.3 Activity and sleep patterns are altered in DopR deficient males

Previous studies have demonstrated a role of DopR in the regulation of sleep and arousal by employing the *dumb<sup>2</sup>* mutant. It has been shown that these flies sleep more but have otherwise comparable activity levels to wild type controls (Lebestky *et al.*, 2009; Ueno *et al.*, 2012). However, these studies were based on *dumb<sup>2</sup>* males. We extended the analyses of sleep and

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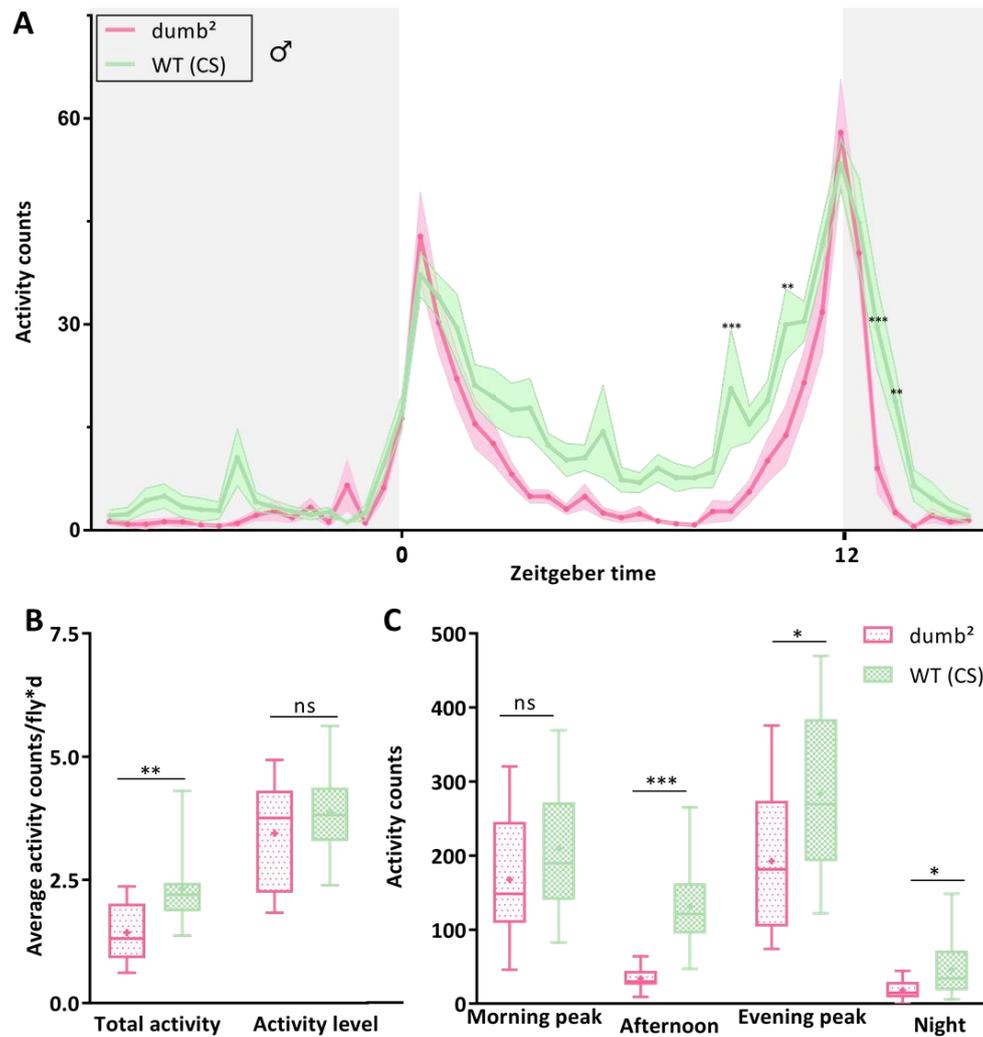
activity patterns to cover *dumb<sup>2</sup>* females and further included additional information on phenotypic details.

### 3.1.3.1 DopR deficiency is associated with decreased total activity in males but not in females

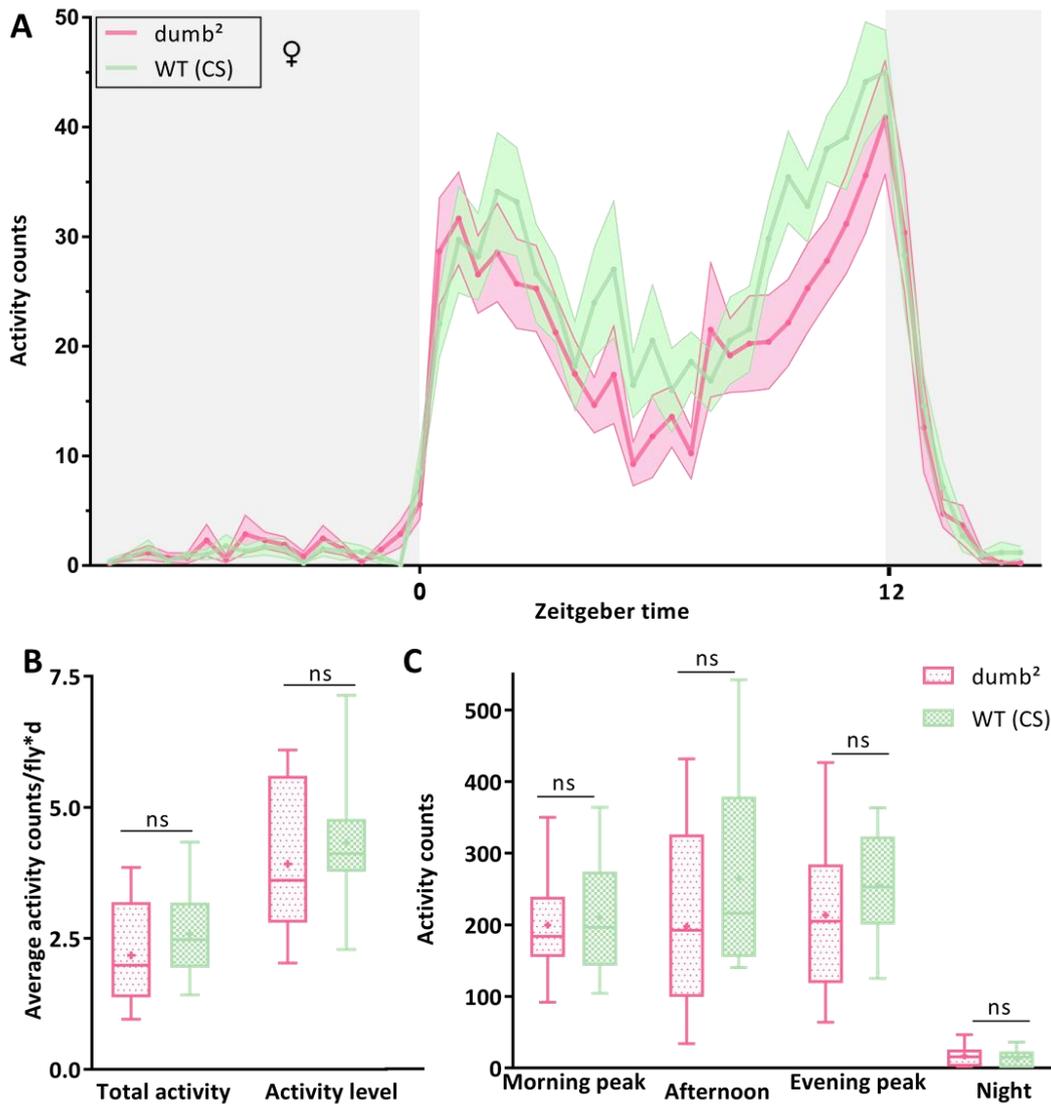
The analysis of activity of *dumb<sup>2</sup>* mutant males could reproduce the findings published by Lebestky *et al.* (2009) and Ueno *et al.* (2012).

DopR deficiency was found to be associated with a 37.5 % reduction in total activity per day (Figure 3.5 B; *dumb<sup>2</sup>* n = 14, WT (CS) n = 15; unpaired t test \*\* p = 0.0015). However, the reduction in overall activity was dependent on the time of the day and did not occur to the same level throughout the day (Figure 3.5 A and C). The pooled activity was affected especially during the afternoon (12-18 zt; unpaired t test \*\*\* p < 0.0001), evening (18-24 zt; unpaired t test \* p = 0.0167) and second half of the night (0-6 zt; Mann Whitney test \*\*\* p < 0.0185) but not changed during the morning phase (6-12 zt; unpaired t test p = 0.2119). Strikingly, the activity of mutants during wake phases was not different to the level observed in control flies, indicating intact locomotor abilities but an increase in rest time (Figure 3.5 B; unpaired t test p = 0.2438).

The total activity was unaffected in *dumb<sup>2</sup>* mutant females as no effect was seen in pooled activity per day (Mann Whitney test p = 0.1816, *dumb<sup>2</sup>* n = 15, WT (CS) n = 13; Figure 3.6 B), per hour (Figure 3.6 A, two-way ANOVA p = 0.5306) or period of the day (Mann Whitney test or unpaired t test p = ns; Figure 3.6 C). Moreover, as in mutant males, the activity level in wake phases was unaffected (unpaired t test p = 0.4262; Figure 3.6 B).



**Figure 3.5 | The total activity is reduced in *dumb<sup>2</sup>* mutant males.** Activity counts of individual flies were recorded with the DAM system (TriKinetics) at 12:12 light-dark cycle and 25°C (day)/20°C (night) on glucose-agar medium. (A) The total activity is plotted over the course of the day. Data points represent means  $\pm$ SEM (two-way ANOVA \*\*\*  $p < 0.0001$ , followed by Bonferroni multiple comparison test). (B) The pooled daily total activity is reduced (unpaired t test \*\*  $p = 0.0015$ ) but activity levels appeared normal (unpaired t test  $p = 0.2438$ ). (C) indicates pooled total activities over day periods. The morning activity levels were unchanged (6-12 zt; unpaired t test  $p = 0.2119$ ), however the strongest reduction was recorded during the afternoon (12-18 zt; unpaired t test \*\*\*  $p < 0.0001$ ) and mild reductions in total activity occurred in the evening (18-24 zt; unpaired t test \*  $p = 0.0167$ ) and second half of the night (0-6 zt; Mann Whitney test \*\*\*  $p < 0.0185$ ). Grey shaded background indicates light-off period. (B and C) Plots illustrate the 25% to 75% percentiles (box), median (horizontal line), mean (plus) and min and max values (whiskers). *dumb<sup>2</sup>*  $n = 14$ , WT (CS)  $n = 15$ . \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.0001$ .



**Figure 3.6 | The total activity is unaffected in *dumb<sup>2</sup>* mutant females.** Activity counts of individual flies were recorded with the DAM system (TriKinetics) at 12:12 light-dark cycle and 25°C (day)/20°C (night) on glucose-agar medium. (A) The total activity is plotted over the course of the day. Data points represent means  $\pm$ SEM (two-way ANOVA  $p = 0.5306$ , followed by Bonferroni multiple comparison test). (B) The pooled daily total activity was unaffected (Mann Whitney test  $p = 0.1816$ ) as well as activity levels of wake phases (unpaired  $t$  test  $p = 0.4262$ ). (C) indicates pooled total activities over day periods (morning = 6-12 zt; afternoon = 12-18 zt; evening = 18-24 zt; night = 0-6 zt; Mann Whitney or unpaired  $t$  tests = ns). Grey shaded background indicates light-off period. (B and C) Plots illustrate the 25% to 75% percentiles (box), median (horizontal line), mean (plus) and min and max values (whiskers). *dumb<sup>2</sup>*  $n = 15$ , WT (CS)  $n = 13$ .

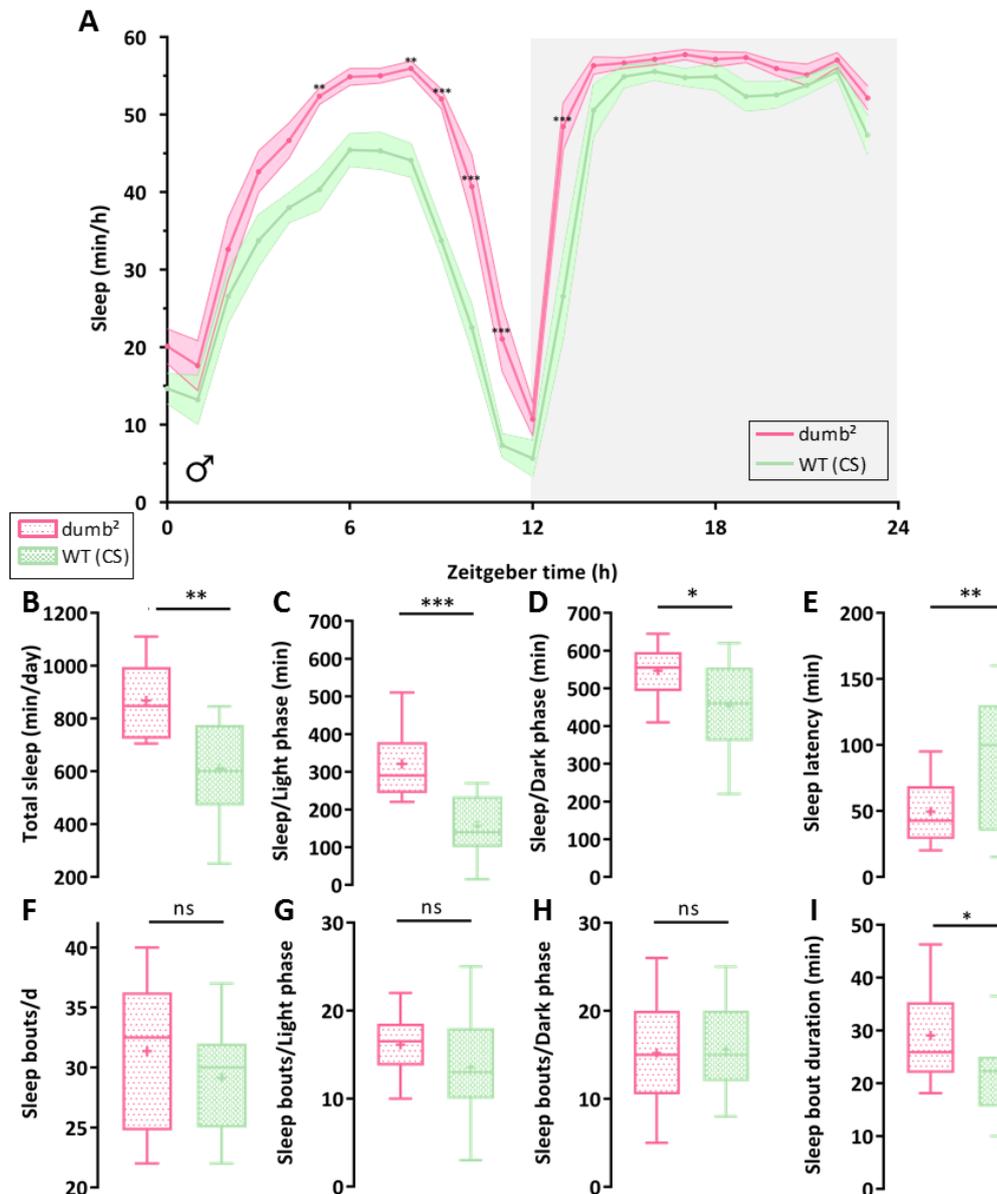
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### 3.1.3.2 DopR deficiency is associated with increased sleep in males but not in females

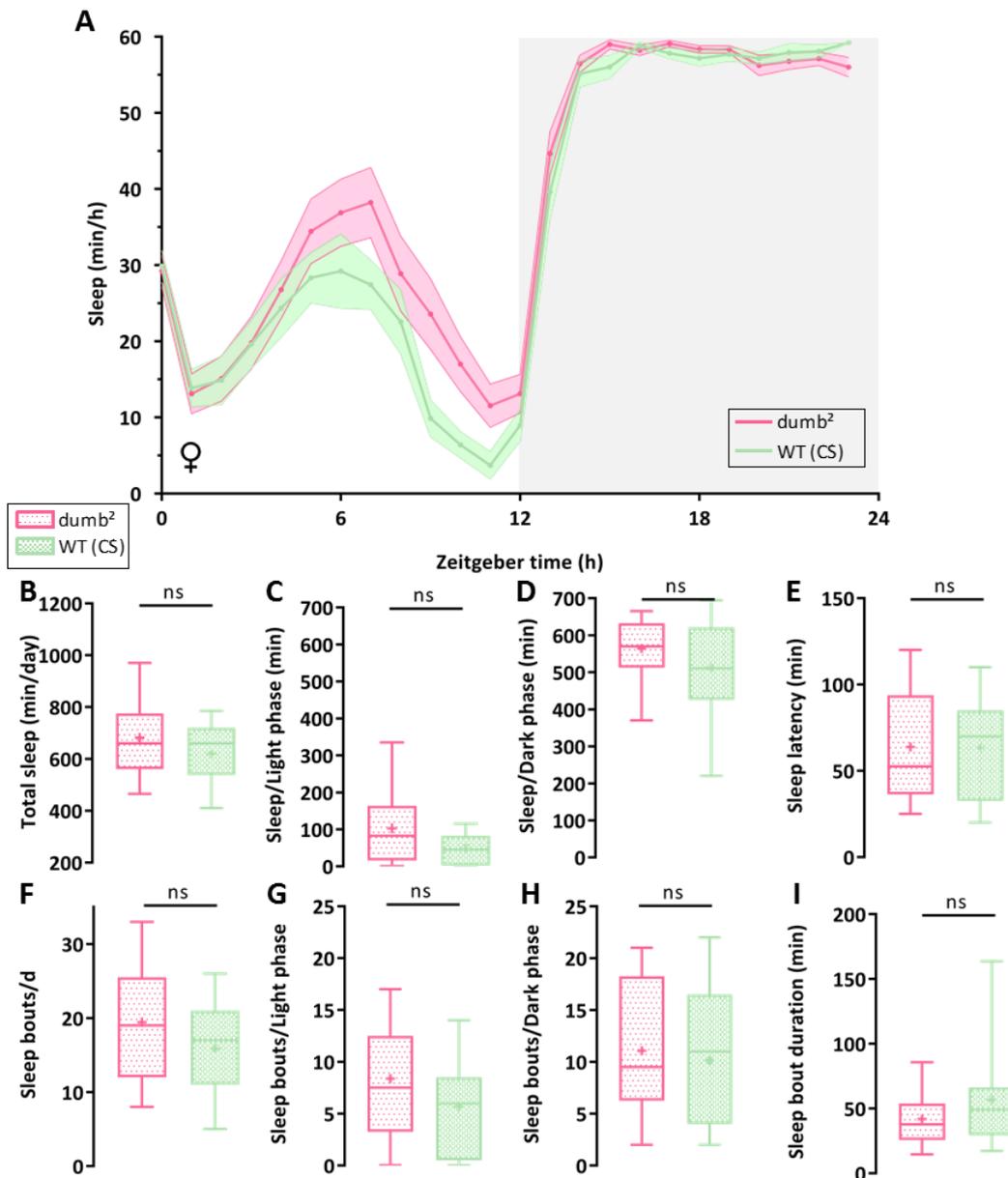
As reported for *dumb<sup>2</sup>* mutants under 3.1.3.1, DopR deficiency is associated with decreased total activity at least in males. To test whether altered sleep levels underlie this reduction, we analysed the sleep pattern of mutants.

The total sleep time was increased by 42 % in DopR knockout males (unpaired t test \*\*  $p = 0.0002$ ; Figure 3.7 A and B; *dumb<sup>2</sup>*  $n = 14$ , WT (CS)  $n = 15$ ). However, the number of sleep bouts appeared unchanged (unpaired t test  $p = 0.2677$ ; Figure 3.7 F, also G and H) but sleep phases were slightly extended by 37 % (Mann Whitney test \*  $p = 0.0447$ ; Figure 3.7 I). Especially daytime sleep was increased (unpaired t test \*\*\*  $p < 0.0001$ ; Figure 3.7 C) although a mild increase in nighttime sleep was also seen (unpaired t test \*  $p = 0.0125$ , Figure 3.7 D). Furthermore, *dumb<sup>2</sup>* mutant males showed reduced sleep latency and went to sleep ~1.8 times quicker than corresponding controls (unpaired t test \*\*  $p = 0.0079$ , Figure 3.7 E).

Female *dumb<sup>2</sup>* mutants did not show altered sleep levels and none of the detailed sleep parameters was affected. Nevertheless, a slight tendency to increased sleep might be indicated in the number of bouts and minutes of sleep during daytime (Figure 3.8 C and G)



**Figure 3.7 | Sleep behaviour of *dumb*<sup>2</sup> mutant males.** Activity counts of individual flies were recorded with the DAM system (TriKinetics) at 12:12 light-dark cycle and 25°C (day)/20°C (night) on glucose-agar medium and 5 min of inactivity were considered as sleep. The circadian sleep pattern is displayed in (A) (two-way ANOVA, followed by Bonferroni multiple comparison test). The total sleep time (B, unpaired t test, \*\**p* = 0.0002) was increased but the number of sleep bouts per day appeared unchanged (F, unpaired t test, \*\*\**p* = 0.2766; see also G and H for sleep bouts number during night or daytime). The increased sleep per day could be primarily caused by intensified daytime sleep (C, unpaired t test, \*\*\**p* < 0.0001) but nighttime sleep was increased as well (D, unpaired t test \* *p* = 0.0125). Moreover, the sleep latency was strongly reduced (E, unpaired t test, \*\**p* = 0.0079). The average sleep bout duration throughout the day was slightly increased (I, Mann Whitney test, *p* = 0.0447). In (A), data points represent means and error bands encompass SEM. Grey shaded background indicates light-off period corresponding to Zeitgeber time 12 - 24. In (B - I), plots illustrate the 25% to 75% percentiles (box), median (horizontal line), mean (plus) and min and max values (whiskers). *dumb*<sup>2</sup> *n* = 14, WT (CS) *n* = 15. \* *p* < 0.05, \*\* *p* < 0.01, \*\*\* *p* < 0.0001.

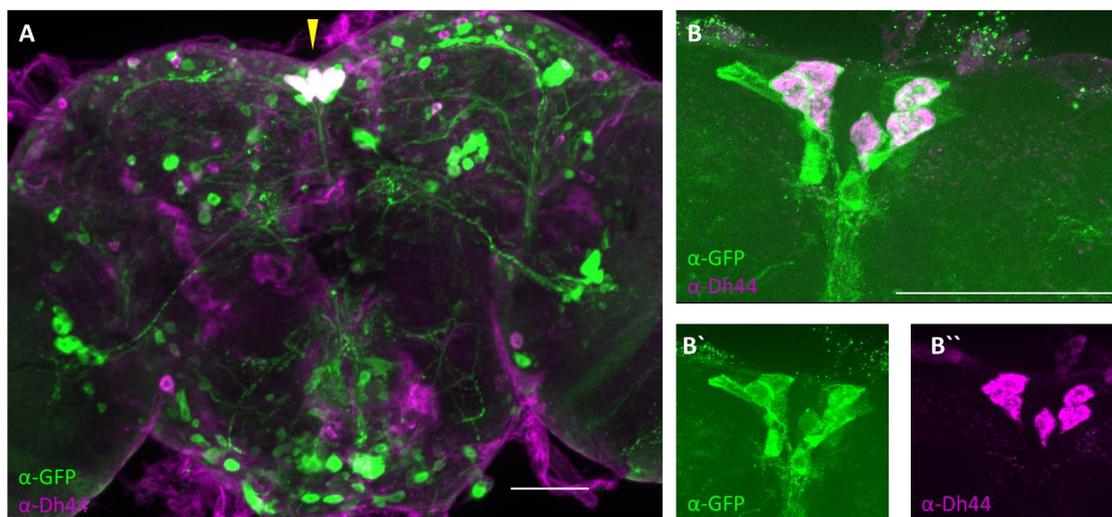


**Figure 3.8 | Sleep behaviour of  $dumb^2$  mutant females.** Activity counts of individual flies were recorded with the DAM system (TriKinetics) at 12:12 light-dark cycle and 25°C (day)/20°C (night) on glucose-agar medium and 5 min of inactivity were considered as sleep. The circadian sleep pattern is displayed in (A). Two-way ANOVA, followed by Bonferroni multiple comparison test revealed no significance. (B) Total sleep time, (F) Number of sleep bouts per day, (C) Minutes of sleep during daytime, (G) Number of sleep bouts during daytime, (D) Minutes of sleep during nighttime, (H) Number of sleep bouts during nighttime, (E) Sleep latency, (I) Average sleep bout duration., 0001. In (A), data points represent means and error bands encompass SEM. Grey shaded background indicates light-off period corresponding to Zeitgeber time 12 - 24. In (B – I), plots illustrate the 25% to 75% percentiles (box), median (horizontal line), mean (plus) and min and max values (whiskers).  $dumb^2$  n = 15, WT (CS) n = 13.

### 3.1.4 DopR as a potential upstream modulator of the diuretic hormone 44 in the *pars intercerebralis*

#### 3.1.4.1 DopR positive cells in the PI synthesise Dh44

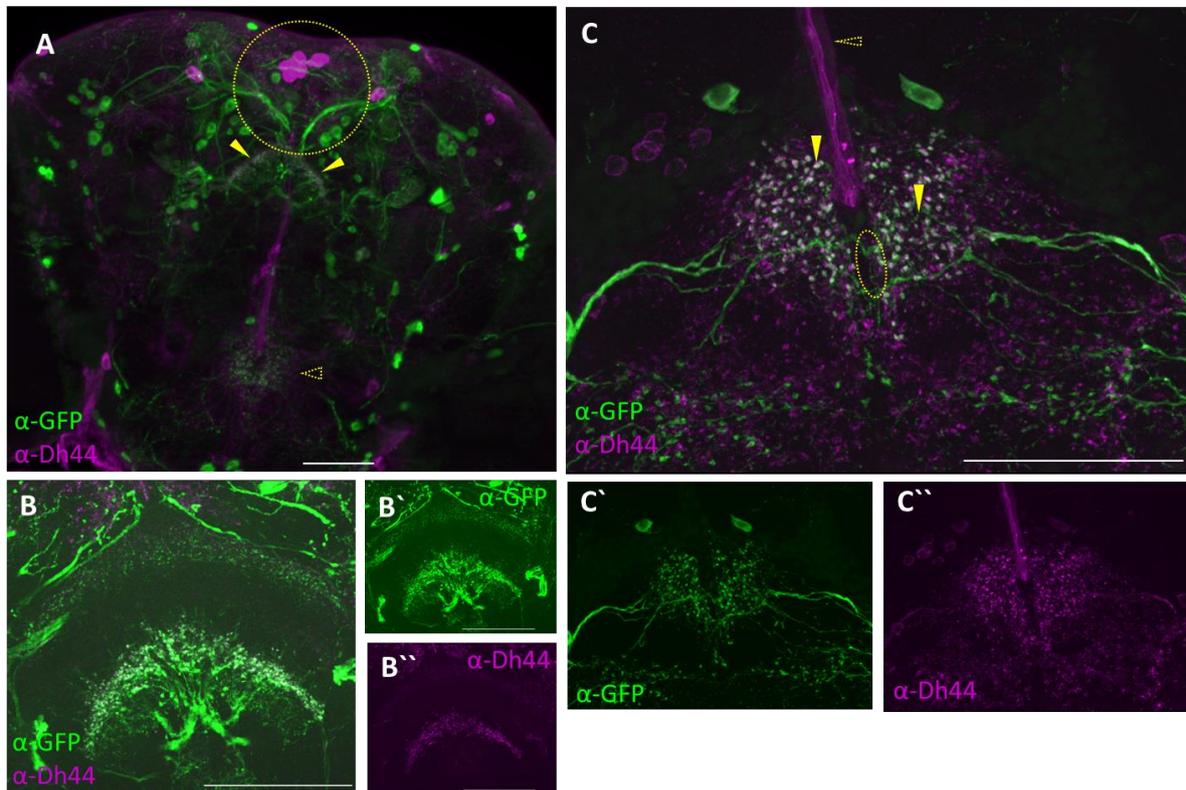
For the purpose of finding leads to potential functional involvements, we have characterised DopR expression pattern in the CNS by employing the DopR-Gal4 line and a GFP reporter (see 3.1.1). By this means, the expression of GFP was targeted to sites of *dopr* promoter activity. As reported above, we have found the DopR to be specifically expressed in the dorsal midbrain, the *pars intercerebralis* (PI), which is part of the neuroendocrine system of the fly and contains neurosecretory cells (de Velasco *et al.*, 2007). Based on the hormone synthesis function of these cells, we supposed a potential modulatory role of the DopR in endocrine function at this site. Further immunohistochemical analysis revealed overlapping expression of DopR and the diuretic hormone 44 (Dh44) in 6 specific cells of the PI (Figure 3.9). Dh44 represents the fly homolog of the mammalian *corticotropin-releasing* factor and is implicated in various physiological processes (further details will be explained in the following sections of this thesis).



**Figure 3.9 | Colocalisation of DopR and Dh44 in six specific cells in the PI.** Immunohistochemical staining of GFP (green) and Dh44 (magenta). GFP visualises *dopr* promoter activity in *dopr > 20xUAS-6xgfp* flies. (A) Overview of the central brain. The colocalisation of DopR (GFP) and Dh44 is detected in the dorsal mid-brain (PI, yellow arrow head; white indicates overlapping signal). (B) Detailed view of the coexpression site in the PI. Note that in all 6 Dh44 positive cells DopR signal could be detected. (B') Detailed view of the GFP signal, indicating DopR expression. (B'') Detailed view of Dh44 IR. Scale bars = 50  $\mu$ m.

Furthermore, we used Gal4-mediated labelling of dopaminergic cells to identify potential DA input sites to Dh44 cells. Brains of flies expressing GFP in the pattern of the *th* gene promoter activity were stained with antibodies against GFP and the Dh44 protein.

Dopaminergic cells were found to arborize extensively in the dorsal midbrain, with processes extending through the whole region or terminating at this site (Figure 3.10). Dh44 cells bodies were literally embedded within this network, which points towards dopaminergic synaptic connections to Dh44 cells directly within the PI region.



**Figure 3.10 | Immunohistochemical co-staining of DA and Dh44 positive neurons.** Antibody staining of GFP (green) and Dh44 (magenta) to reveal potential dopaminergic input sites to Dh44 cells. GFP visualises the promoter activity in the  $> 20xUAS-6xgfp$  flies, labelling dopaminergic neurons. (A) Overview of GFP signals and Dh44 IR in the central brain. Extensive dopaminergic arborisations were labelled within the PI, implying DA input to Dh44 cells in this region (circle). Filled arrow heads indicate the fan-shaped body, in which overlapping GFP signal and Dh44 IR was detected (enlarged in B, close-up views in B' and B''). Dotted line arrow head indicates overlapping DA signal and Dh44 IR surrounding the oesophagus foramen, see also (C; close-up views in C' and C''); circle indicates oesophagus foramen). Overlapping GFP and Dh44 signal within the fan-shaped body and the oesophagus region most likely indicate Dh44 downstream or converging associations of both messenger systems. Scale bars = 50 μm.

This combined immunohistochemical analysis of DA and Dh44 cells revealed additional, potential Dh44 downstream or converging associations of the hormone with DA. For instance, overlapping signals of Dh44 IR and GFP were detected in the fan-shaped body of the central complex and in the oesophagus region (Figure 3.10 B and C).

### 3.1.4.2 Dopamine induces cAMP generation and Dh44 release from PI cells

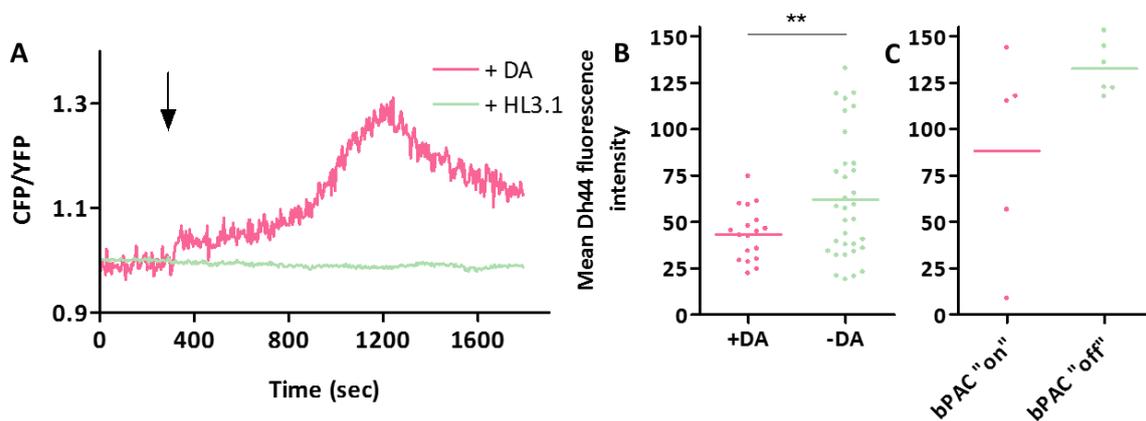
As a basis for further experiments, the functionality of DopR in Dh44 PI cells needed to be established.

DopR has previously been shown to positively interact with AC and to generate cAMP upon receptor activation by DA or appropriate agonists (Sugamori *et al.*, 1995). Thus, the cellular response of the Dh44 PI cells was expected to imply a rise in the cAMP level. To test this, cAMP live cell imaging was performed on ex vivo brains by employing Dh44-GAL4-mediated expression of the genetic fluorescent cAMP indicator UAS-Epac-camps. Epac-coupled CFP and YFP fluorescence intensities were monitored over time and rises in cAMP concentration were indicated by increasing CFP/YFP ratios (decrease in FRET signal).

Of 7 brains tested, each responded to DA application by a decrease in FRET signal and showed a typical curve shape of CFP/YFP ratios indicating cAMP response, which was not present upon application of control medium (HL 3.1). It was thus concluded that a cellular mechanism generates cAMP in PI cells as response to DA exposure. Among the brains tested, the responses varied in intensity and time of onset (Appendix Figure 3). An example curve is shown in Figure 3.11 A.

We further tested the effect of DA exposure on the Dh44 content of PI cells. Brains were dissected and incubated in 1 mM of DA (+DA) or control buffer (-DA) for 30 min. They were subsequently stained with a direct antibody against Dh44 and IR was determined. The average Dh44 IR was diminished by 31 % in brains that were previously exposed to DA, which indicates a release of the hormone stimulated by DA (Figure 3.11 B ; unpaired t test with Welch's correction \*\*  $p = 0.0069$ ; +DA;  $n = 18$  ROIs (8 brains); -DA;  $n = 33$  (9 brains)).

In addition, we artificially increased cAMP levels in Dh44 PI cells by expressing the blue-light activated adenylate cyclase bPAC. Brains of *dh44 > bPAC* flies were dissected and exposed to blue light ex vivo to stimulate cAMP generation by bPAC. The intensity of direct Dh44 IR was subsequently determined, assuming that a cAMP-induced release of Dh44 would decrease mean Dh44 fluorescence intensities. The results might indicate a trend towards lowered Dh44 IR signal in PI cells of flies with increased cAMP levels (Figure 3.11 C), however, based on the low sample size this conclusion is vague.



**Figure 3.11 | Dopamine elicits a cAMP response and Dh44 release from PI cells.** To establish DopR function in PI cells, cAMP live cell imaging was performed and Dh44 IR was quantified to assess hormone release after bath application of DA to ex vivo brains. (A) illustrates an example curve of elicited cAMP response, representing one brain. CFP and YFP fluorescence intensities were recorded from the PI of *dh44 > epac-camps* flies. In the course of the experiments, brains were first exposed to control buffer (HL 3.1) and subsequently to 1 mM DA. Arrow indicates the time point of exposure start. (B) and (C) show individual mean fluorescence intensities of Dh44 IR in the PI after (1) ex vivo incubation with 1 mM DA (+DA;  $n = 18$  ROIs (8 brains)) or control buffer (-DA;  $n = 33$  (9 brains)), which indicate Dh44 release as response to DA application (unpaired  $t$  test with Welch's correction \*\*  $p = 0.0069$ ) and (2) upon activation of bPAC (bPAC "on") in PI cells to enhance cAMP levels (bPAC "off" represents the control condition), which indicates a trend towards Dh44 release in response to bPAC stimulation. Horizontal lines represents the mean across samples. Fluorescence intensities are given in a.u. on the y-axis.

### 3.1.4.3 Manipulation of the cAMP in Dh44 cells to mimic DopR activity

To further test the physiological relevance of DopR signalling in the modulation of Dh44 hormonal function, we chose to artificially manipulate the cAMP level in these cells, thereby mimicking receptor activation but circumventing the dependency on physiological stimuli. An appropriate means in this approach is represented by the optogenetic effector bPAC. bPAC is a blue light inducible AC that is employed in combination with a GAL4 driver line. By using the driver *Dh44<sup>VT</sup>-Gal4* that specifically drives UAS activation in Dh44 PI cells (Cavanaugh *et al.*, 2014, see Appendix Figure 2), we could target bPAC expression specifically to this site and induce cAMP generation by applying blue light.

Dh44 is involved in various physiological processes. To evaluate a potential upstream regulatory role of the DopR in Dh44 signalling, we analysed phenotypes connected with these known functions. The following paragraphs address the potential upstream modulatory role of DopR

in Dh44 signalling in the regulation of rest:activity rhythms (Cavanaugh *et al.*, 2014), the regulation of fluid secretion (Cabrero *et al.*, 2002; Cannell *et al.*, 2016), the evaluation of the nutritive value of sugars and the modulation of intestinal activity (Dus *et al.*, 2015).

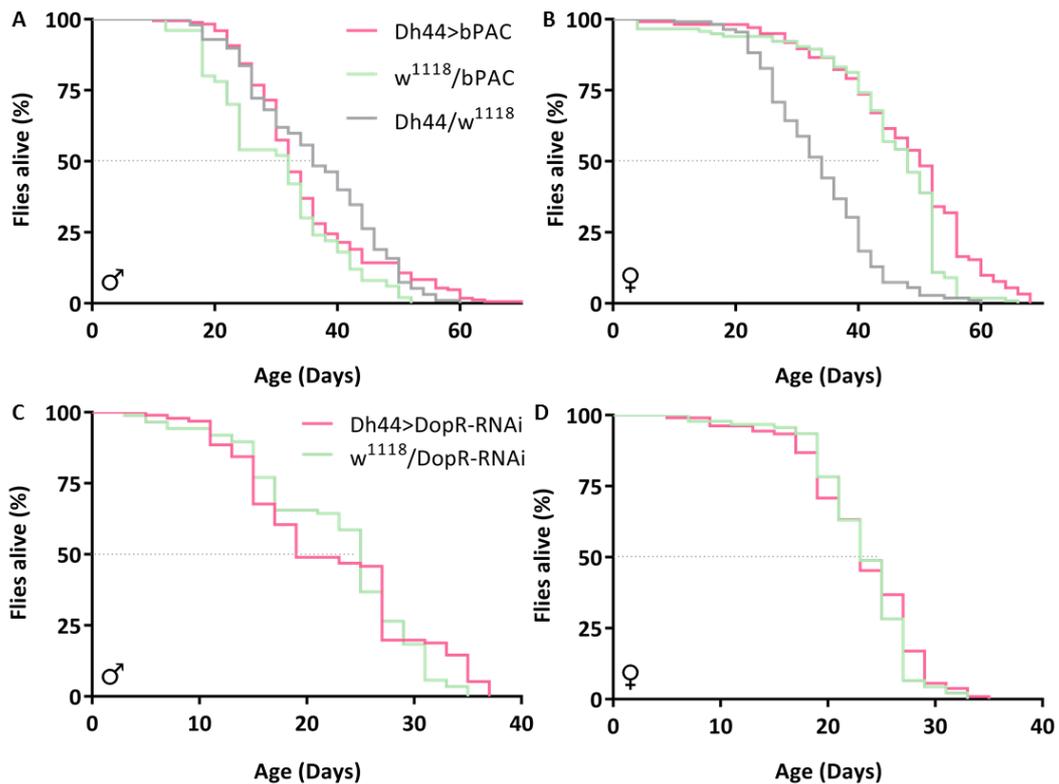
#### 3.1.4.3.1 *DopR* functioning in Dh44 signalling is not essential

To obtain an overview on the physiological relevance of DopR function in Dh44 cells of the PI, we first examined potential effects on the general health of the flies by lifespan and metabolic rate analyses.

The lifespan analyses was conducted by manipulating the potential DopR effect in two directions, (1) by enhancing the effect via bPAC-mediated cAMP generation and (2) by diminishing the effect via the targeted RNAi-mediated knockdown of *dopr*. However, both manipulation modes could not reveal a substantial role of the receptor in the Dh44 producing cells of the PI, neither in male nor in female flies (Figure 3.12).

Male flies that were expected to show an enhanced effect through bPAC-mediated higher cAMP levels suffered a slight reduction in median lifespan but only when compared to the driver line control (Figure 3.12 A; *Dh44>bPAC*, median lifespan: 34 d, n = 5, 124 flies in total; *w<sup>1118</sup>/UAS-bPAC*, median lifespan 32 d, n = 5, 115 flies in total; *Dh44-Gal4/w<sup>1118</sup>*, median lifespan 36 d, n = 5, 125 flies in total; log-rank test \* p = 0.0360). The opposing phenotype was observed in females, as *Dh44>bPAC* flies showed an increase in the median lifespan when compared to the driver line control but not in comparison to the effector control (Figure 3.12 B; *Dh44>bPAC*, median lifespan: 50 d, n = 5, 103 flies in total; *w<sup>1118</sup>/UAS-bPAC*, median lifespan 48 d, n = 5, 118 flies in total; *Dh44-Gal4/w<sup>1118</sup>*, median lifespan 34 d, n = 5, 124 flies in total; log-rank test \*\*\* p < 0.0001).

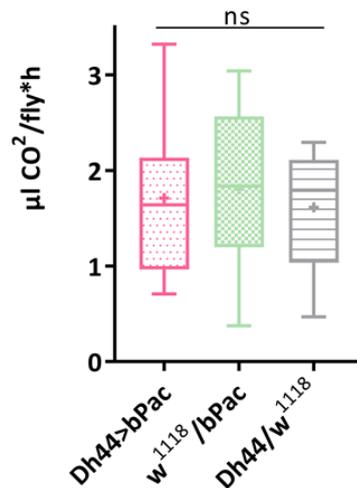
Neither male nor female flies with diminished DopR expression in Dh44 cells were affected in their lifespan (Figure 3.12 C and D; males: *dh44 > DopR-dsRNA*, median lifespan: 19 d, n = 2, 96 flies in total; *w<sup>1118</sup>/UAS-DopR-dsRNA*, median lifespan 25 d, n = 2, 87 flies in total; log-rank test p = 0.2746; females *dh44>DopR-dsRNA*, median lifespan: 23 d, n = 2, 106 flies in total; *w<sup>1118</sup>/UAS-DopR-dsRNA*, median lifespan 23 d, n = 2, 92 flies in total; log-rank test p = 0.3513).



**Figure 3.12 | Lifespan is not affected by high levels of cAMP or DopR depletion in Dh44 PI cells.** The cAMP level was artificially increased by Gal4-mediated expression of the blue light activated adenylate cyclase bPAC under a 12hrs:12hrs blue light/dark cycle. The lifespan did not appear altered in both sexes at least in comparison to the effector control (A – males: Dh44>bPAC, median lifespan: 34 d, n = 5, 124 flies; w1118/UAS-bPAC, median lifespan 32 d, n = 5, 115 flies; Dh44-Gal4/w1118, median lifespan 36 d, n = 5, 125 flies; log-rank test \* p = 0.0360; B – females: Dh44>bPAC, median lifespan: 50 d, n = 5, 103 flies in total; w1118/UAS-bPAC, median lifespan 48 d, n = 5, 118 flies in total; Dh44-Gal4/w1118, median lifespan 34 d, n = 5, 124 flies in total; log-rank test \*\*\* p < 0.0001). In addition, diminishing the potential DopR effect in these cells by RNAi-mediated knockdown of the receptor did not affect the life expectancy of flies (C – males: dh44>DopR-dsRNA, median lifespan: 19 d, n = 2, 96 flies in total; w1118/UAS-DopR-dsRNA, median lifespan 25 d, n = 2, 87 flies in total; log-rank test p = 0.2746; D – females: dh44>DopR-dsRNA, median lifespan: 23 d, n = 2, 106 flies in total; w1118/UAS-DopR-dsRNA, median lifespan 23 d, n = 2, 92 flies in total; log-rank test p = 0.3513). The proportion of alive flies is plotted in % over time. Dotted lines indicate median survival times.

A further general overview on the metabolic constitution of flies with a potentially enhanced DopR effect in Dh44 cells, was obtained by respirometric determination of the metabolic rates. In this assay CO<sub>2</sub> production is analysed as a measure for O<sub>2</sub> consumption and thus the level of metabolism (Yatsenko *et al.*, 2014).

However, metabolic rates of *Dh44>bPAC* males did not appear different from the rates of corresponding controls under blue light exposition (one-way ANOVA  $p = 0.8061$ ; Figure 3.13).



**Figure 3.13 | CO<sub>2</sub> production is unaffected in flies with high levels of cAMP in Dh44 cells.** Metabolic rates of males expressing the blue light activated AC bPAC in Dh44 PI cells were assessed by respirometric determination of CO<sub>2</sub> production under blue light exposure. The assay could not reveal a change in metabolic rates of *Dh44>bPAC* flies (one-way ANOVA  $p = 0.8061$ , followed by Tukey's multiple comparison test = ns; *Dh44>bPAC*  $n = 11$ , *w<sup>1118</sup>/UAS-bPAC*  $n = 17$ , *Dh44-Gal4/w<sup>1118</sup>*  $n = 12$ ) Plots illustrate the 25% to 75% percentiles (box), median (horizontal line), mean (plus) and min and max values (whiskers).

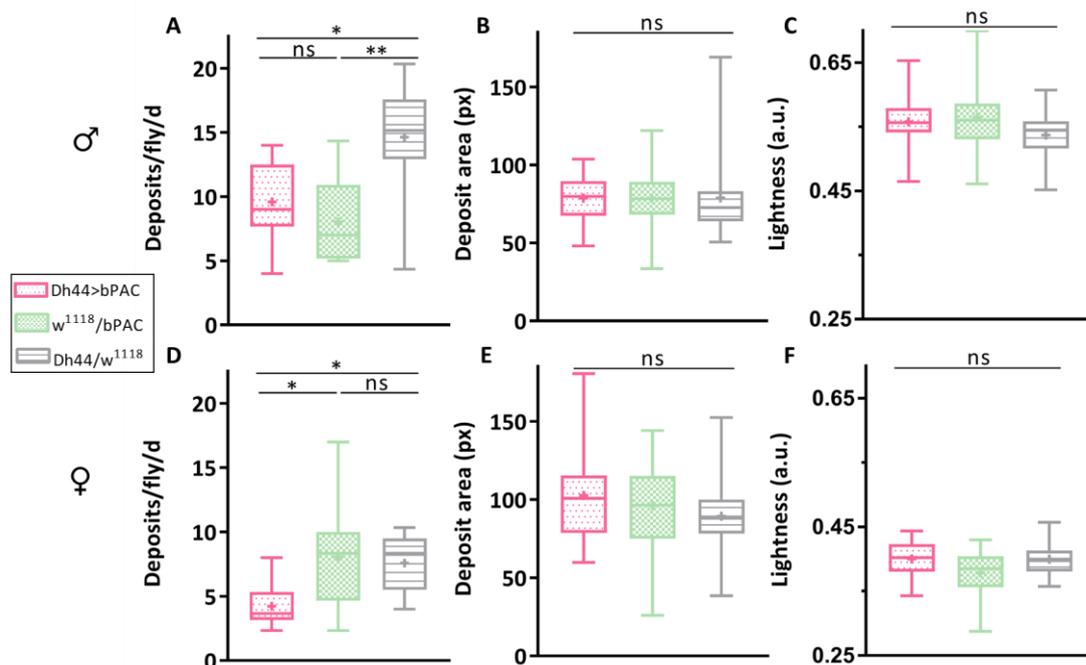
### 3.1.4.3.2 The cAMP level in Dh44 cells modulates excretion and desiccation tolerance

*Dh44* has been shown to stimulate excretion and correlating gut motility (Dus *et al.*, 2015). Moreover, as the name implies, the hormone acts as a modulator of fluid secretion in the principal cells of Malpighian tubules (Cabrero *et al.*, 2002). We combined the testing for involvement of DopR in both physiological processes, waste excretion and fluid secretion, in one read-out by analysing the number and further graphical characteristics of excreta (Cognigni, Bailey and Miguel-Aliaga, 2011; Wayland *et al.*, 2014). In parallel to intestinal function, diuresis rates were assessed from colour lightness values as these correlate with the water content of excreta (please refer to methods section for further details).

*Dh44>bPAC* males did not show significant changes in their excretion rate when compared to the *w<sup>1118</sup>/UAS-bPAC* effector control although the overall comparison of all genotypes revealed significant differences (one-way ANOVA \*\*  $p = 0.0027$ , followed by Tukey's multiple comparison test as indicated in Figure 3.14 A; *Dh44>bPAC*  $n = 9$ , *w<sup>1118</sup>/UAS-bPAC*  $n = 8$ , *Dh44-*

*Gal4/w<sup>1118</sup>* n = 10). The deposit sizes as well as lightness values indicated normal diuresis (Figure 3.14 B and C; *Kruskal-Wallis test* = ns), which is additionally supported by circularity levels (not shown).

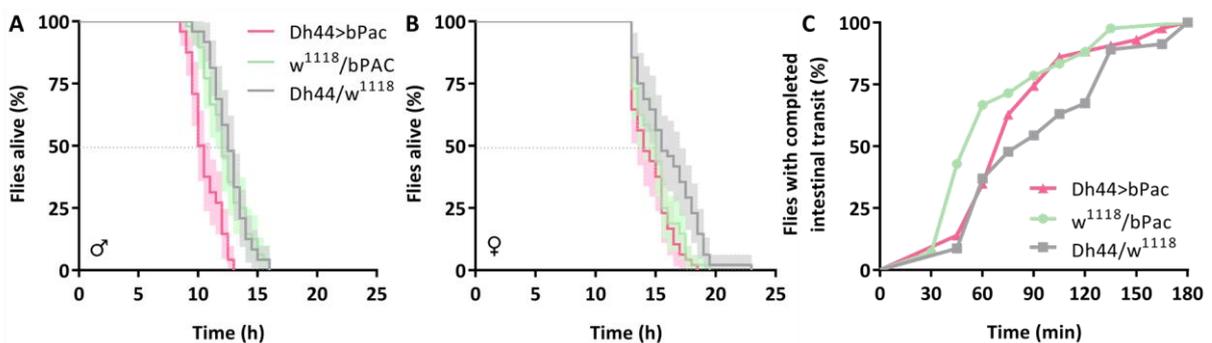
However, faecal output was reduced in *Dh44>bPAC* females (one-way ANOVA \* p = 0.0170, followed by Tukey's multiple comparison test as indicated in Figure 3.14 D; *Dh44>bPAC* n = 10, *w<sup>1118</sup>/UAS-bPAC* n = 10, *Dh44-Gal4/w<sup>1118</sup>* n = 9), which represents the opposite effect to the Dh44-mediated stimulation of excretion described by Dus *et al.* (2015). Diuresis was not affected in females as well, as inferred from deposit size information, lightness of spots (Figure 3.14 E and F; one-way ANOVA and *Kruskal-Wallis test* = ns) and spot circularity (not shown).



**Figure 3.14 | cAMP in Dh44 PI cells affects the excretion rate of females.** To assess the excretory phenotype of *Dh44>bPAC* flies, groups of three were kept in vials with dyed food and cover slips onto which the flies placed their faecal deposits. After 24 hours, the cover slips were analysed by employing a software tool to extract the graphical information of excreta (Wayland *et al.*, 2014). Neither the excretion rate (one-way ANOVA \*\* p = 0.0027, followed by Tukey's multiple comparison test – results depicted in A), nor the size and lightness of spots appeared altered in *Dh44>bPAC* males (B and C; *Kruskal-Wallis test* = ns; *Dh44>bPAC* n = 9, *w<sup>1118</sup>/UAS-bPAC* n = 8, *Dh44-Gal4/w<sup>1118</sup>* n = 10). The number of faecal spots was reduced in *Dh44>bPAC* females (one-way ANOVA \* p = 0.0170, followed by Tukey's multiple comparison test – results depicted in D) but there was no indication for altered diuresis from the size and lightness information of spots (E and F; one-way ANOVA and *Kruskal-Wallis test* = ns; *Dh44>bPAC* n = 10, *w<sup>1118</sup>/UAS-bPAC* n = 10, *Dh44-Gal4/w<sup>1118</sup>* n = 9) Plots illustrate the 25% to 75% percentiles (box), median (horizontal line), mean (plus) and min and max values (whiskers). \* p < 0.05, \*\* p < 0.01.

As a diuretic hormone, Dh44 stimulates fluid secretion from Malpighian tubules (Cabrerero *et al.*, 2002) and related functions in desiccation and osmotic stress tolerance have been described (Cannell *et al.*, 2016; Hector *et al.*, 2009). To further test the effect of elevated cAMP levels in Dh44 cells in this regard, we analysed the tolerance to desiccation stress of *Dh44>bPAC* flies. Moreover, we determined intestinal transit times in order to test for a modulatory function in gut motility (with regard to the finding by Dus *et al.* (2015)).

Desiccation tolerance was assessed by recording the survival times of flies that were deprived of food and water under constant blue light conditions to activate bPAC in PI cells. *Dh44>bPAC* males were significantly less tolerant to desiccation stress as the median survival time was reduced by approximately 14.5 % (from 12 h and 12.5 h for *w<sup>1118</sup>/UAS-bPAC* (n = 48) and *Dh44-Gal4/w<sup>1118</sup>* (n = 48) control flies to 10.25 hours in *Dh44>bPAC* flies (n = 48); Figure 3.15 A, log-rank test \*\*\* p < 0.0001). However, when *Dh44>bPAC* females were tested the sensitivity to desiccation stress appeared significantly increased only in comparison to the driver line control (median survival times: 15 h and 15.5 h for *w<sup>1118</sup>/UAS-bPAC* (n = 48) and *Dh44-Gal4/w<sup>1118</sup>* (n = 48) control flies to 14 hours in *Dh44>bPAC* flies (n = 48); Figure 3.15 B, log-rank test \*\* p = 0.0033).



**Figure 3.15 | cAMP in Dh44 cells increases sensitivity to desiccation but does not affect intestinal transit.**

For the determination of desiccation tolerance, flies were deprived of food and water and exposed to blue light to activate bPAC in Dh44 cells. (A) The desiccation sensitivity was increased in Dh44/bPAC males (log-rank test \*\*\* p < 0.0001, n = 48 for each genotype) but not different when Dh44/bPAC females were compared to the w<sup>1118</sup>/UAS-bPAC control, although the overall log-rank test was significant (\*\* p = 0.0033, n = 48 for each genotype, B). (C) The expression and activation of bPAC in Dh44 PI cells did not affect the intestinal transit time as no specific trend could be revealed for the Dh44>bPAC females. Nevertheless, the data points appeared different in log-rank analysis (\* p = 0.0217; Dh44/bPAC n = 43, w<sup>1118</sup>/UAS-bPAC (n = 42), Dh44-Gal4/w<sup>1118</sup> n = 46). Error bands encompass 95 % CI. Dotted lines indicate median survival times.

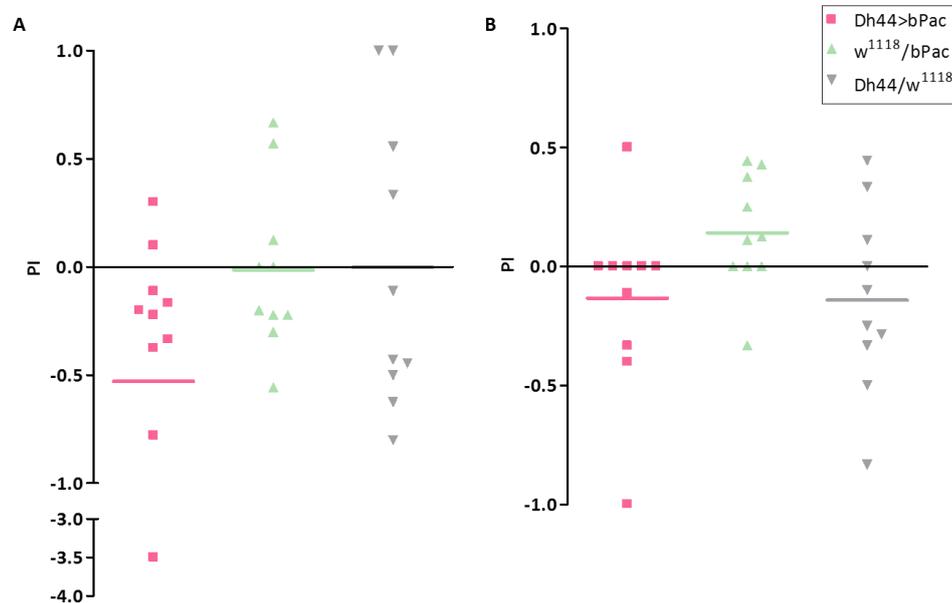
Regarding the intestinal transit times, no effect was observed when *Dh44>bPAC* females were compared to controls, although the overall log-rank test appeared significant with \* p = 0.0217

(Figure 3.15 C; *Dh44>bPAC* flies (n = 43), *w<sup>1118</sup>/UAS-bPAC* (n = 42) and *Dh44-Gal4/w<sup>1118</sup>* (n = 46)).

### 3.1.4.3.3 The nutrient sensor function is not affected by cAMP in PI cells

Dus *et al.* (2015) found evidence for the role of the Dh44 system in post-ingestive, taste-independent nutrient sensing in PI cells that is involved in feeding circuits. Disturbed Dh44 expression or cellular function underlie the inability of flies to distinguish between nutritive and non-nutritive sugars.

In order to clarify a potential role of cAMP in the nutrient sensor function of the Dh44 system, a choice assay was performed to test the ability of *Dh44>bPAC* flies to evaluate the nutritivity of sugars. D-fructose (nutritive) was tested against D-arabinose (non-nutritive) and the flies were allowed to eat for 2 hours under constant blue light exposure to activate bPAC. Preference indices (PI) indicated no preference for either sugar, consistent for *Dh44>bPAC* flies and corresponding control genotypes as well as for both sexes (Figure 3.16). Nevertheless, in comparison to controls, at least male *Dh44>bPAC* flies showed a tendency to prefer the non-



**Figure 3.16 | cAMP is not affecting the nutrient sensor function of the Dh44 system.** *Dh44>bPAC* flies were starved and subsequently served with differently coloured D-fructose and D-arabinose containing media to assess their preference for these nutritive and non-nutritive sugars. The assay was performed for two hours under constant blue light exposure to activate the bPAC transgene. (A) Male flies might show a tendency to prefer arabinose but female *Dh44>bPAC* flies performed comparable to their corresponding controls (B). PI = preference index; n = 10, 10 flies each.

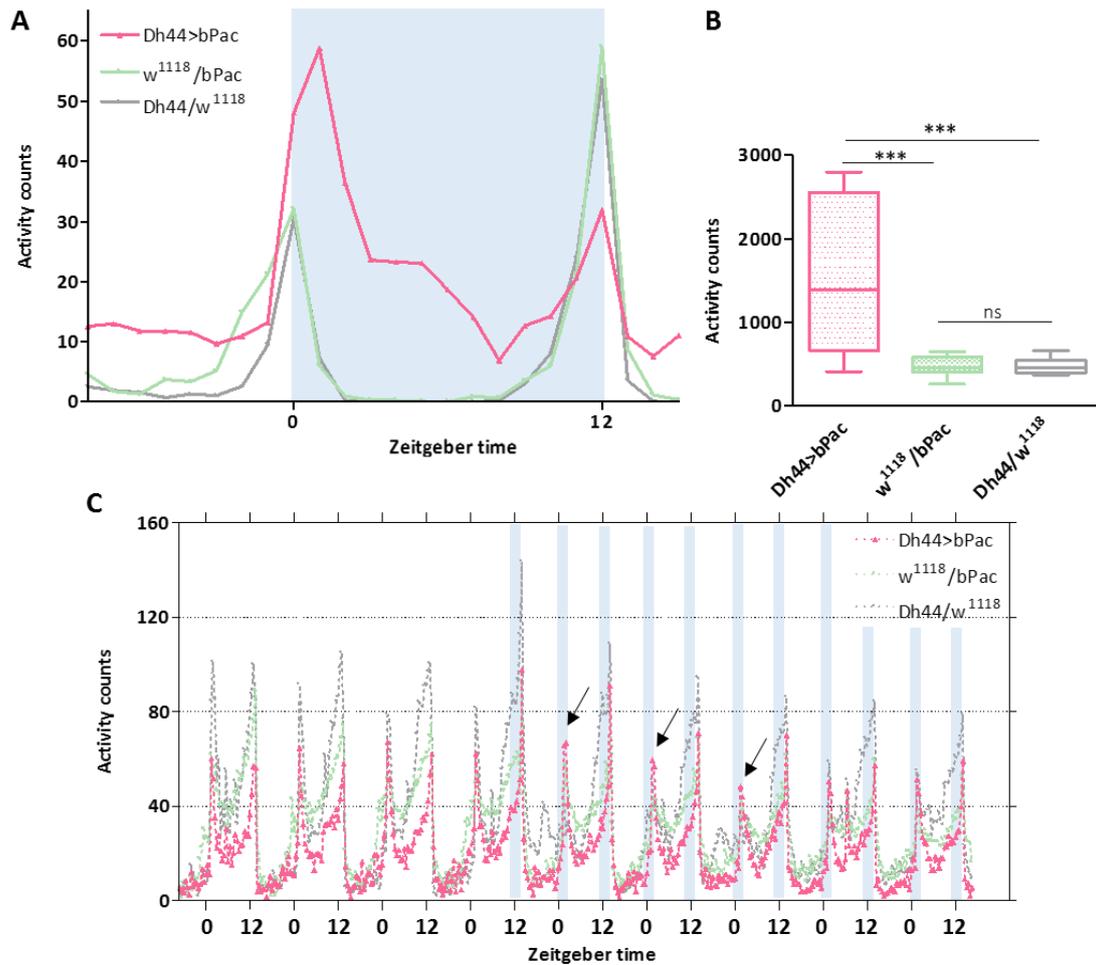
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nutritive but sweeter arabinose over fructose, indicating a potential minor role, if any, of cAMP in PI cells to influence the Dh44 nutrient sensor system.

#### 3.1.4.3.4 *The cAMP level in Dh44 cells modulates the fly's activity*

Besides other functions, Dh44 acts as a central circadian output molecule. Dh44 cells are in direct contact with clock neurons, thus receiving circadian clock information; removal of the Dh44 protein or constant activation of its synthesising cells were described to cause arrhythmicity (Cavanaugh *et al.*, 2014). To examine a potential upstream regulatory role of the DopR in this process, we manipulated the cAMP level and analysed the activity pattern.

The expression and activation of bPAC in PI cells did not decrease fly rhythmicity but significantly increased the general activity level (Figure 3.17). This phenotype was reproducible under different bPAC activation regimes. In a first approach, flies were constantly exposed to blue light for 12 consecutive hours, which caused the flies to be 3 times more active than corresponding control flies (one-way ANOVA \*\*\* $p < 0.0001$ ,  $n = 10$  each; Figure 3.17 A and B). In a second approach, we exposed the flies to 30 min. blue light pulses within their morning and evening peaks of activity (Figure 3.17 C). The flies were first entrained to a temperature cycle in constant darkness and the first blue light pulse was set within the evening peak of the fourth day of entrainment. As it becomes apparent in the activity graph the flies responded to this first blue light application by a rise in activity, which is attributable to the light effect itself. However, on the following 3 days *dh44>bPac* flies showed enhancement of the morning peak activity, which was not detected in corresponding controls. Nevertheless, this effect vanished on the fourth day of blue light application. Interestingly, consistent for both blue light regimes, only the morning peak of activity was enhanced, whereas the evening peak was attenuated.



**Figure 3.17 | Activity levels are modulated by cAMP signalling in PI cells.** Locomotor activity of *dh44>bPAC* males was monitored under two different bPAC activation regimes by blue light exposure. (A) Flies were exposed to blue light for a period of 12 hours per day (shaded area), which significantly enhanced activity levels. (B) Sum of activity counts illustrated in (A). The total activity counts were strongly elevated in *dh44>bPAC* flies (one-way ANOVA followed by Tukey's multiple comparison test, see graph,  $n = 10$  for each genotype). (C) Flies were entrained to a temperature cycle for 3 days and exposed to 30 min of blue light within their morning and evening peaks of activity, starting from the evening peak at day 4 (shaded areas indicate blue light pulses). *dh44>bPAC* flies showed enhanced morning peak activity at the first 3 days of the light pulse regime (arrows). Activity curves indicate mean activity values of genotype groups (A and C). In (B), plots illustrate the sums of activity counts seen in (A); 25% to 75% percentiles (box), median (horizontal line), min and max values (whiskers). \*\*\*  $p < 0.0001$ .

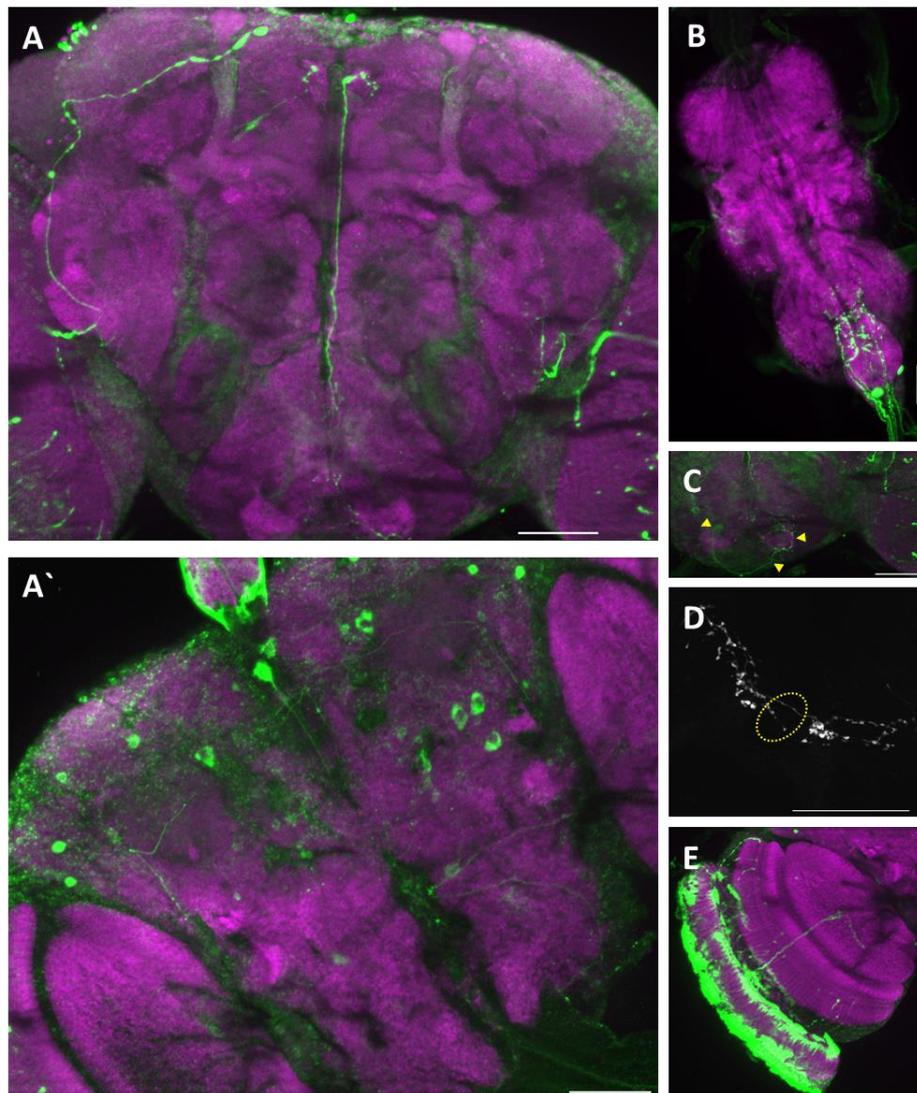
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## 3.2 Role of D2R in metabolism and activity regulation

### 3.2.1 *d2r* promoter activation pattern in the brain and VNC

The *d2r* promoter activation pattern in the CNS was analysed in *d2r-Gal4 > 20xUAS-6x-gfp* flies. The GFP signal revealed single cell bodies located in different brain regions as well as, in some cases, strong and long processes extending throughout the brain. In the anterior central brain (Figure 3.18 A) several cell bodies were located in the dorsal midbrain (*pars intercerebralis*), from which processes extended ventrally along the midline. These extensions as well as processes presumably coming from the lateral peripheries ramified in the region of the oesophagus foramen (Figure 3.18 A, A' and D). Furthermore, long neuronal processes originating in the *pars intercerebralis* were extending laterally towards the optic lobes. Additional green labelled processes are associated with the protocerebral bridge of the central complex (Figure 3.18 A').

In the anterior brain, a GFP labelled network was associated with mushroom body  $\alpha$  and  $\alpha'$  lobes (Figure 3.18 A). Additionally, specific neuronal processes were associated with substructures in the gnathal ganglion (Figure 3.18 C). Very strong *d2r* promoter activation was seen in the optic lobes, especially in the lamina and in labelled photoreceptor cells terminating within the medulla. Moreover, *d2r* promoter activation was detected in the Hofbauer-Buchner tract (Figure 3.18 E, see also section 4.4). Within in the VNC, a fishbone-like meshwork was labelled in the abdominal ganglion (Figure 3.18 B).

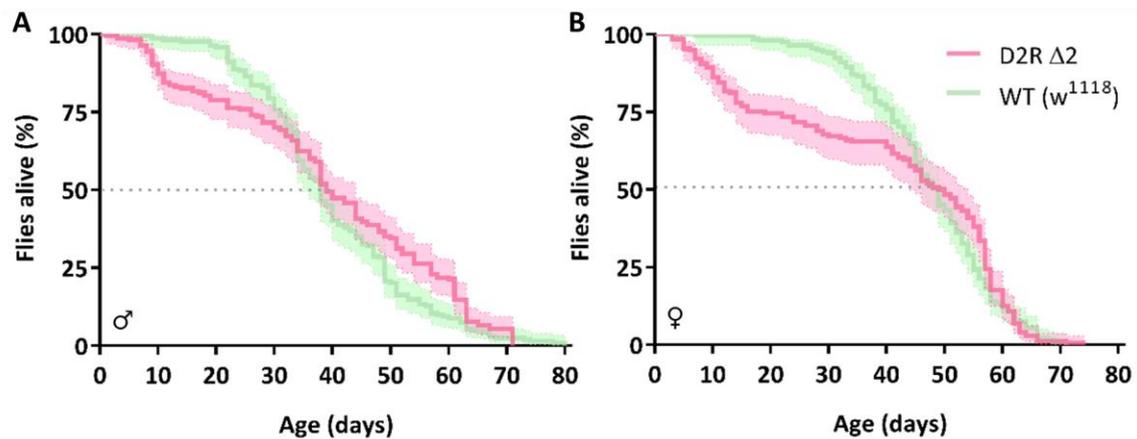


**Figure 3.18 | *d2pr* promoter activation pattern in the adult CNS.** Immunohistochemical analysis of GFP reporter signal (white and green) in *d2r-Gal4 > 20xUAS-6xgfp* flies.  $\alpha$ -NC82 costaining of neuropil regions is shown in magenta. GFP signal associated with *d2r* promoter activation was detected in cell bodies and neuronal processes throughout different regions of the brain and VNC. (A) Overview of *d2r* expression in the anterior central brain. GFP signals located in the posterior brain are shown in (A'). GFP labelled cell bodies were present in the *pars intercerebralis*, from which processes extended ventrally and towards the lateral peripheries; in association with the PB. The OL showed strong signal (E), especially within the L and in photoreceptor cells that penetrate into the ME. Moreover, the Hofbauer-Buchner tract was strongly labelled. GFP labelled processes were also found at the ventral tip of the GNG (C, arrow heads) and surrounding the oesophagus foramen (indicated as circle, D). The VNC showed a fishbone-like meshwork within the abdominal ganglion (B). Scale bars = 50  $\mu$ m.

### 3.2.2 D2R deficient flies suffer metabolic impairments

#### 3.2.2.1 D2R mutant flies show age-dependent variations in survival probabilities

To determine the overall severity of D2R deficiency on the health state of the flies, I examined the lifespan. The log-rank analyses across the entire survival curves did not reveal a difference



**Figure 3.19 | Age-dependent survival probabilities in D2R deficient flies.** The lifespan assay was performed at 25°C/20°C light/dark cycle (12hrs:12hrs) on standard medium. The proportion of alive flies is plotted in % over time. (A) Mutant male flies die at a significantly higher frequency within the first 34 d post eclosure, whereas the risk of death is decreased at older age. (B) Survival of mutant female flies is decreased within the first 46 d post eclosure. The curves were split at their crossing points for statistical analysis (see text). Dotted lines indicate median survival times. Error bands indicate 95 % CI.

between the D2R mutants and corresponding controls, neither for males nor for females. For males, median survival was determined as 39 days for mutants ( $n = 7$ , total 223 flies) and 38 days for control flies ( $n = 7$ , total 228 flies) (Figure 3.19 A, log-rank test  $p = 0.1008$ ). A median survival of 50 days was recorded for mutant female flies ( $n = 7$ , total 196 flies) and 49 days for control females ( $n = 7$ , total 220 flies) (Figure 3.19 B, log-rank test  $p = 0.9345$ ). Moreover, the maximum lifespans were not different for D2R knockout male flies (D2R  $\Delta 2$  ♂, max. lifespan:  $67.18 \text{ d} \pm 3.75 \text{ sd}$ ; wild type ♂, max. lifespan  $65.78 \text{ d} \pm 6.921 \text{ sd}$ ; Mann Whitney test  $p = 0.1489$ ) or D2R knockout female flies (D2R  $\Delta 2$  ♀, max. lifespan:  $64.05 \text{ d} \pm 3.268 \text{ sd}$ ; wild type ♀, max. lifespan  $64.77 \text{ d} \pm 3.054 \text{ sd}$ ; Mann Whitney test  $p = 0.3003$ ).

However, the Kaplan-Meier curves indicated non-proportional hazard ratios with a higher probability of death for mutants at younger age when compared to wild type flies. This pattern was observed for male and female flies (Figure 3.19). For further statistical analyses, the curves

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were split at their crossing points and the two “survival” phases (SP) were analysed separately (Appendix Figure 4).

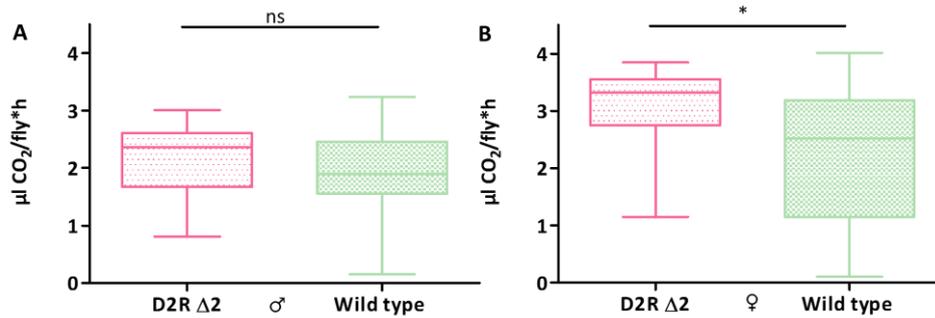
For male flies survival phase 1 (SP1) corresponds to days 1-34 and SP2 to days 35-80 after eclosion (Figure 3.19 A and Appendix Figure 4 A and B). In SP1, the median lifespan of mutants was significantly shortened by 40% with an HR of 1.897 (D2R  $\Delta 2$  ♂, SP1 median lifespan: 18 d; wild type ♂, SP1 median lifespan 30 d; log-rank test \*\*\*  $p < 0.0001$ ). On the contrary, in SP2, median lifespan of D2R knockout flies was increased by 8% with an HR of 0.7576 when compared to control flies (D2R  $\Delta 2$  ♂, SP2 median lifespan: 51 d; wild type ♂, SP2 median lifespan 47 d; log-rank test \*  $p = 0.018$ ).

For female flies SP1 corresponds to days 1-46 and SP2 to days 47-74 after eclosion (Figure 3.19 B and Appendix Figure C and D). In SP1, the median lifespan of mutants was significantly shortened by 60% with an HR of 1.931 (D2R  $\Delta 2$  ♀, SP1 median lifespan: 16 d; wild type ♀, SP1 median lifespan 40 d; log-rank test \*\*\*  $p < 0.0001$ ). The median lifespan of female D2R knockout flies in SP2 was not different from wild type control flies (D2R  $\Delta 2$  ♀, SP2 median lifespan: 57 d; wild type ♀, SP2 median lifespan 55 d; log-rank test  $p = 0.1367$ ).

#### 3.2.2.2 D2R deficiency leads to elevated metabolic activity in female flies

To obtain further insight into the metabolic constitution of flies lacking D2R, I determined the metabolic rates respirometrically.

Figure 3.20 depicts produced CO<sub>2</sub> per fly and hour. D2R knockout flies show elevated levels of CO<sub>2</sub> production, although this only appears significant for female mutants with an average increase of 25% when compared to corresponding control flies.

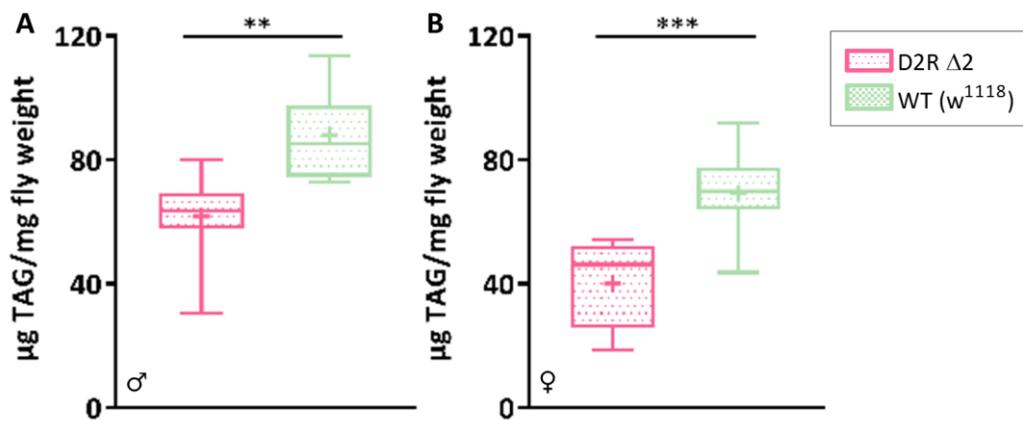


**Figure 3.20 | CO<sub>2</sub> production is elevated in D2R mutant flies.** Metabolic rates of D2R knockout flies were assessed by respirometric determination of CO<sub>2</sub> production. (A) Male mutants show an elevated but not significant level of CO<sub>2</sub> production per hour (Mann Whitney test  $p = 0.2845$ , D2R  $\Delta 2$   $n = 20$ , wild type = WT (w1118)  $n = 22$ ). (B) Female mutants produced significantly more CO<sub>2</sub> per hour than corresponding controls, indicating an increased metabolic rate (Mann Whitney test  $p = 0.0294$ , D2R  $\Delta 2$   $n = 20$ , wild type = WT (w1118)  $n = 22$ ). Plots illustrate the 25% to 75% percentiles (box), median (horizontal line) and min and max values (whiskers). \*  $p < 0.05$ .

### 3.2.2.3 D2R deficiency is associated with lower body fat levels

In animals, metabolic energy is reserved in the form of triacylglycerols (TAG) and glycogen in order to be able to maintain homeostasis whenever energy is consumed. In general, caloric yield is higher from stored TAGs than from carbohydrates or proteins. Differences in body fat levels of flies raised under same constant conditions might indicate impairments in lipid metabolism and thus energy imbalances. To examine whether the lipid homeostasis is affected in D2R mutants, I determined the total TAG content of knockout and wild type flies by using the coupled colorimetric assay (Figure 3.21) (Hildebrandt *et al.*, 2011).

Both, male and female D2R knockout flies, appeared leaner when compared to corresponding controls. The total TAG content in male mutants is reduced by 30 % (Figure 3.21 (A); unpaired t test  $p = 0.0005$ ). However, female mutants suffered from a more intense phenotype with a 42% decrease in TAG level when compared to wild type controls (B); unpaired t test  $p < 0.0001$ ).



**Figure 3.21 | Total TAG content is lower in D2R deficient flies.** 5 days old flies were transferred to high fat diet for 8 days and the total TAG level was determined by using the coupled colorimetric assay. (A) Male D2R mutant flies contain less TAG per mg weight than corresponding controls (unpaired t test, \*\*  $p = 0.0005$ ,  $n = 10$  each). The same phenotypic effect was observed in female flies as D2R mutants showed a significantly decreased TAG content (unpaired t test, \*\*\*  $p < 0.0001$ ,  $n = 11$  each). Plots illustrate the 25% to 75% percentiles (box), median (horizontal line), mean (plus) and min and max values (whiskers). \*\*  $p < 0.01$ , \*\*\*  $p < 0.0001$ .

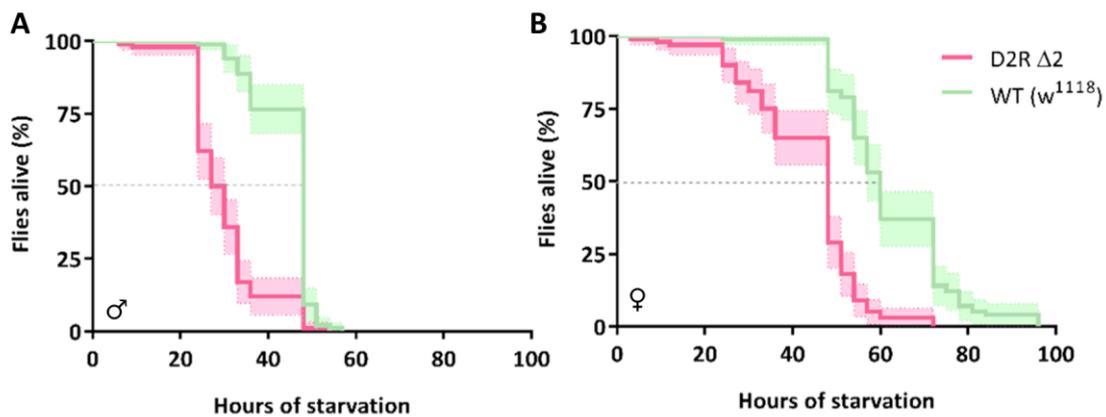
#### 3.2.2.4 D2R deficient flies are less resistant to starvation

The ecological condition of longer periods of food deprivation represents a stress to affected organisms, in which energy reserves are mobilised to meet the energy demand. In these cases, amongst other regulatory mechanisms, sufficient energy storage is required to maintain homeostatic balance. Thus, flies with insufficient energy reserves appear less resistant to starvation and die earlier under starvation conditions than flies with unimpaired metabolism. As D2R deficient flies showed lower TGA levels than wild type flies we examined the severity of the potential energy imbalances by testing the capability of the mutants to resist against starvation.

For the assay, flies were raised on standard medium at 25°C. Ten vials were prepared per group, each with a 2% agar bottom and water-soaked filter paper to prevent desiccation. 5-8 days old flies were assayed in groups of ten.

Both, male and female D2R knockout flies, appeared less tolerant against food deprivation than corresponding controls. The median lifespan of male mutants dropped by 41% when compared to wild type males with an HR of 2.853 (Figure 3.22 A) (D2R  $\Delta 2$  ♂, median lifespan: 28.5 h; wild type ♂, median lifespan 48 h; log-rank test \*\*\*  $p < 0.0001$ ;  $n = 10$  each, in total 100 flies per group). In the comparison of mutant and wild type females, the HR was determined as 2.924 and the median lifespan of knockout flies was reduced by 20% to 48 h (Figure 3.22 B) (wild

type ♀, median lifespan 60 h; log-rank test \*\*\*  $p < 0.0001$ ;  $n = 10$  each, in total 100 flies per group).



**Figure 3.22 | Starvation resistance is reduced in flies deficient of the D2R.** 5-8 days old flies were assayed in groups of ten. Agar bottom vials were equipped with wet filter paper and dead flies were counted every three hours. The diagrams display the proportion of alive flies in % over time. Under starvation conditions, mutant male flies appeared less resistant than control flies with a 41 % reduction in their median lifespan (A; D2R  $\Delta 2$  ♂, median lifespan: 28.5 h; wild type ♂, median lifespan 48 h; log-rank test \*\*\*  $p < 0.0001$ ;  $n = 10$  each). As in male flies, the median lifespan of mutant female flies was reduced by 20% (B; D2R  $\Delta 2$  ♀, median lifespan: 48 h; wild type ♀, median lifespan 60 h; log-rank test \*\*\*  $p < 0.0001$ ;  $n = 10$  each). Dotted lines indicate median survival times. Error bands indicate 95 % CI.

### 3.2.2.5 D2R deficiency does not affect the intestinal physiology

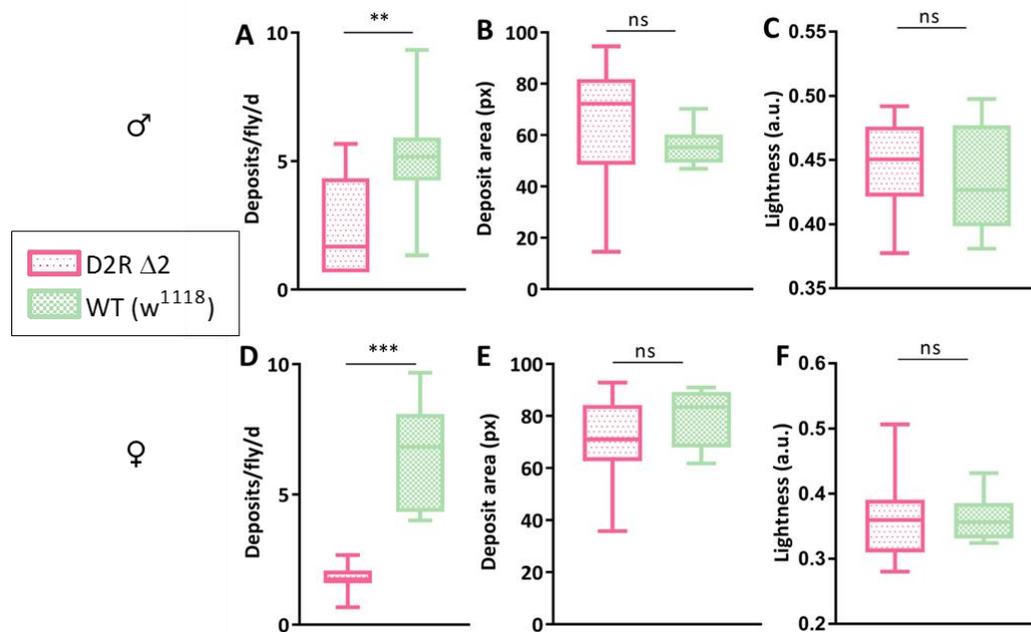
The gastrointestinal tract (GIT) is the central supplier organ system, which processes the up-taken food to facilitate nutrient supply to the organism. The intestinal physiology is thus crucial in the maintenance of body homeostasis. A previous study has demonstrated GFP signal, indicating *d2r* promoter activation, in intestinal stem cells as well as enteroblasts (Stephano, 2013). Since we detected several metabolic abnormalities in D2R knockout flies, we subsequently investigated the physiological integrity of the gut as an impaired intestinal function might underlie the observed metabolic phenotypes.

#### 3.2.2.5.1 Excreta analyses indicate an intact intestinal physiology of D2R deficient flies

The further analyses of the intestinal integrity was based on graphical characteristics of excreta (Cognigni, Bailey and Miguel-Aliaga, 2011). For this assay, fly food vials were prepared with blue-dyed standard medium. In addition, a cover slip was placed into the vial onto which flies excreted and which was later scanned for a detailed analysis of the deposits. A software tool

was employed to extract information on size, shape and gray values of faecal spots (Wayland *et al.*, 2014).

Faecal output was assessed by the number of faecal deposits on the cover slips. As illustrated in Figure 3.23 (A and D) both male and female D2R mutants show a reduction in total faecal output by 52% and 73% respectively (D2R  $\Delta 2$  ♂, unpaired t test  $**p = 0.008$ ,  $n = 10$ ; D2R  $\Delta 2$  ♀, unpaired t test with Welch's correction  $***p < 0.0001$ ,  $n = 10$ ). This phenotype is generally observed when flies suffer from an impaired GIT physiology (Martorell *et al.* 2014) but is moreover associated with food intake (Cognigni *et al.*, 2011).



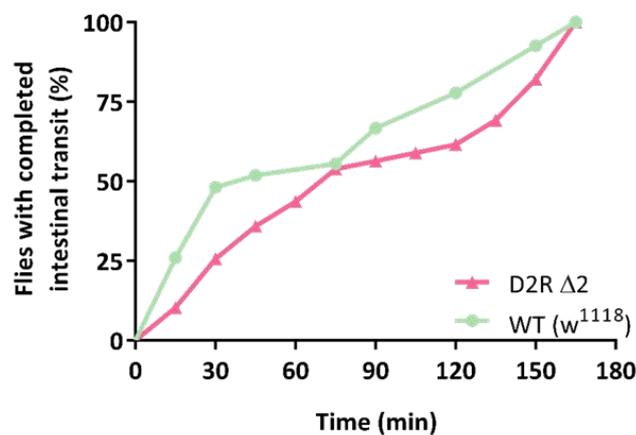
**Figure 3.23 | D2R knockout flies show reduced faecal output but otherwise normal excretion.** To assess the excretory phenotype of D2R mutants, groups of three flies were placed in vials with dyed food and cover slips. After 24 hours, the cover slips were analysed with a software tool (T.U.R.D, (Wayland *et al.*, 2014a)). Although the total faecal output is reduced in both male mutants (A, unpaired t test  $**p = 0.008$ ,  $n = 10$ ) and female mutants (D, unpaired t test with Welch's correction  $***p < 0.0001$ ,  $n = 10$ ) other diagnostic traits, such as size (males B, females E) and lightness (males C, females F) are not affected. Plots illustrate the 25% to 75% percentiles (box), median (horizontal line) and min and max values (whiskers).  $** p < 0.01$ ,  $*** p < 0.0001$ .

However, other diagnostic parameters, such as deposit size and colour lightness, indicative of water content, are not affected in both sexes (Figure 3.23, males B and C, females E and F). In addition, shape, optical density and the correlation of size and lightness are not different from control flies (not shown).

### 3.2.2.5.2 D2R deficiency does not affect the intestinal transit time

A reduced number of excreta might be associated with a prolonged intestinal transit (Martorell *et al.*, 2014). As reported in the previous section, D2R deficient flies show a decrease in the number of faecal deposits consistent for both males and females. To test whether the reduction in faecal output of mutants is due to an extended intestinal transit time, I determined the time to first excretion events of flies feeding on dyed food. Due to methodical reasons, only the transit time of females was measured.

The intestinal transit time of D2R mutant females was not different from the transit time of wild type controls (Figure 3.24) (log-rank test  $p = 0.1789$ ; D2R  $\Delta 2$   $n = 39$ , wild type  $n = 27$ ).

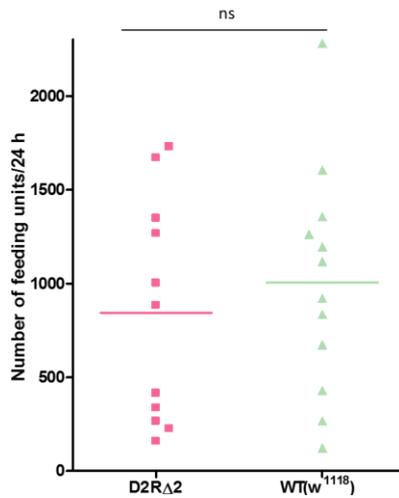


**Figure 3.24 | D2R deficiency does not affect the intestinal transit time.** Within a time frame of 3 hours, the time to the first excretion event was determined for female flies. To enhance the visibility of excreta, the flies were fed on dyed food. No difference could be detected between the gut transit times of D2R mutant and wild type flies (log-rank test  $p = 0.1789$ ; D2R  $\Delta 2$   $n = 39$ , wild type  $n = 27$ ).

### 3.2.2.6 Food intake is unimpaired in D2R mutants

To test whether the reduction in body fat levels and the decrease in starvation tolerance are attributable to a general reduction in food intake, feeding events were quantified by employing the Fly Liquid Food Interaction Counter (Ro *et al.*, 2014).

Based on the results of the FLIC, the feeding occurs to a normal rate in D2R mutants (unpaired  $t$  test  $p = 0.5278$ ,  $n \geq 11$ ), indicating no difference in food intake between mutants and controls (Figure 3.25).



**Figure 3.25 | Food intake is not reduced in D2R  $\Delta$ 2 mutants.**

The feeding rate was determined by employing the FLIC system. The number of feeding units was unchanged in D2R mutants (unpaired t test  $p = 0.5278$ ,  $n \geq 11$ ). Horizontal lines indicate the mean, symbols indicate the sum of feeding units over the course of 24 h.

### 3.2.3 Activity and sleep patterns are altered in D2R deficient flies but rhythmically stable

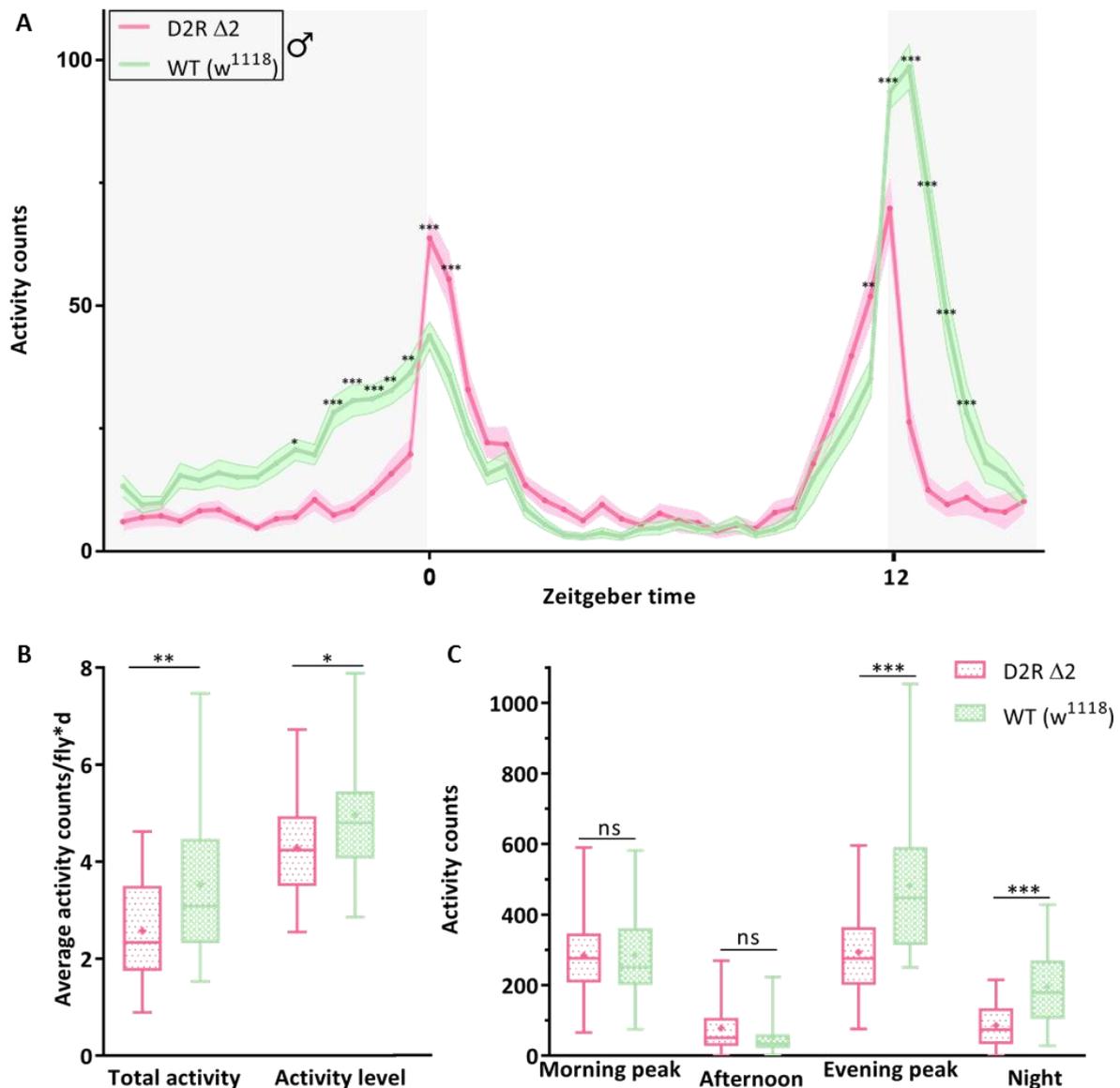
Draper *et al.* (2007) used an RNAi approach and could demonstrate reduced locomotor activity in flies with diminished D2R levels. To further test the involvement of the D2R in fly activity control and patterning, I examined the activity level, sleep and rhythmic behaviour of D2R knockout flies. To rule out the possibility that the observed phenotypes are caused by the knock-out of the gene *CG17003*, I examined the activity pattern of flies with an ubiquitous knockdown of the gene; no relevant phenotype was observed (Appendix Figure 5).

#### 3.2.3.1 D2R deficiency is associated with decreased total activity

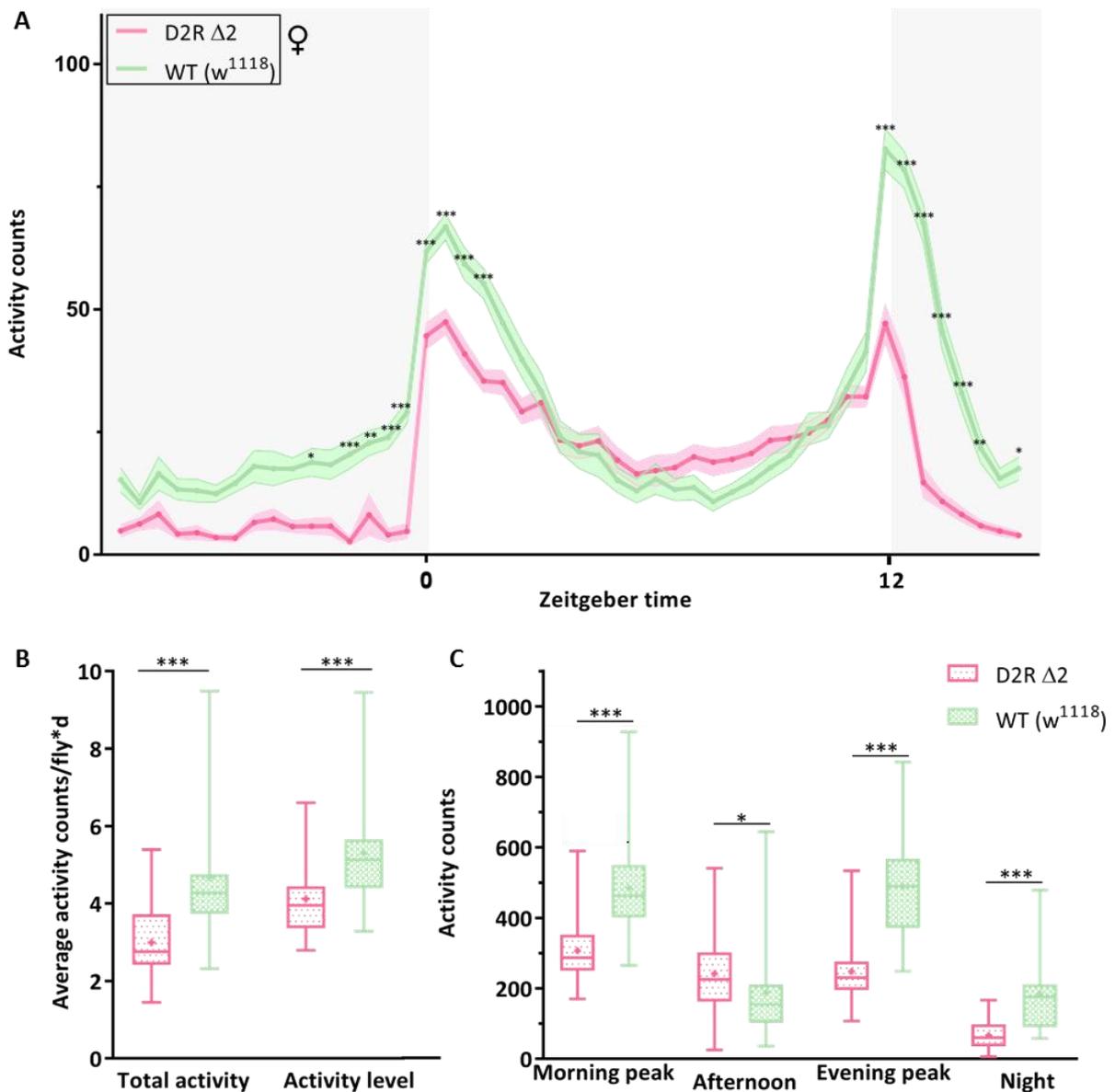
Principally, the D2R deficiency of knockout flies was associated with impaired total activity and intensity levels throughout the day for male and female flies (Figure 3.26 and 3.27).

As illustrated in Figure 3.26 B, male D2R mutants were generally less active than wild type flies as the daily total activity amount was reduced by 27% (Mann Whitney test,  $**p = 0.006$ ; D2R  $\Delta$ 2  $n = 28$ , WT (w<sup>1118</sup>)  $n = 32$ ). In wake phases, however, the actual activity level appeared to be reduced to a lesser extent although it still appeared significant (reduction by 14%; Mann Whitney test,  $*p = 0.0417$ ; D2R  $\Delta$ 2  $n = 28$ , WT (w<sup>1118</sup>)  $n = 32$ ). Less activity of mutant flies was predominantly detected in the evening peak and night phase (Figure 3.26 A and C) but also

in the period before the maximum of the morning peak. However, within the first hour of light, the activity of male D2R mutants exceeded the activity of wild type flies (see Figure 3.26 A).



**Figure 3.26 | The total activity is reduced in D2R  $\Delta 2$  mutant males.** Activity counts of individual flies were recorded with the DAM system (TriKinetics) at 12:12 light-dark cycle and 25°C (day)/20°C (night) on glucose-agar medium. (A) The total activity is plotted over the course of the day. Data points represent means  $\pm$ SEM (two-way ANOVA \*\*\*  $p < 0.0001$ , followed by Bonferroni multiple comparison test). (B) The pooled daily total activity is reduced (unpaired t test \*\*  $p = 0.006$ ) as well as activity levels but to a lesser extent (unpaired t test \*  $p = 0.0417$ ). (C) indicates pooled total activities over day periods. The morning and afternoon activity levels were unchanged (6-12 zt and 12-18 zt; unpaired t test  $p = ns$ ) and the strongest reduction was recorded during the evening and night (18-24 zt and 0-6 zt, Mann Whitney test \*\*\*  $p < 0.0001$ ). Shaded background indicates light-off period. (B and C) Plots illustrate the 25% to 75% percentiles (box), median (horizontal line), mean (plus) and min and max values (whiskers). D2R  $\Delta 2$   $n = 28$ , WT (CS)  $n = 32$ . \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.0001$ .



**Figure 3.27 | The total activity is reduced in D2R  $\Delta 2$  mutant females.** Activity counts of individual flies were recorded with the DAM system (TriKinetics) at 12:12 light-dark cycle and 25°C (day)/20°C (night) on glucose-agar medium. (A) The total activity is plotted over the course of the day. Data points represent means  $\pm$ SEM (two-way ANOVA \*\*\*  $p < 0.0001$ , followed by Bonferroni multiple comparison test). (B) As it was found for male mutants, the pooled daily total activity is reduced (Mann Whitney test \*\*\*  $p = 0.0001$ ) as well as activity levels but to a lesser extent (Mann Whitney test \*\*\* $p = 0.0003$ ). (C) indicates pooled total activities over day periods. During the morning, evening and night phases, activity was generally reduced. However, during the afternoon, mutants showed higher activity levels than controls. Shaded background indicates light-off period. (B and C) Plots illustrate the 25% to 75% percentiles (box), median (horizontal line), mean (plus) and min and max values (whiskers). D2R  $\Delta 2$   $n = 28$ , WT (CS)  $n = 32$ . \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.0001$ .

Figure 3.27 depicts the general activity characteristics of female D2R mutants. Similar to male mutants, female mutants were generally less active than wild type flies. The daily total activity amount was reduced by 36% (Mann Whitney test, \*\*\* $p = 0.0001$ ; D2R  $\Delta 2$   $n = 26$ , WT ( $w^{1118}$ )  $n = 32$ ) and the activity level by 22% (Mann Whitney test, \*\*\* $p = 0.0003$ ; D2R  $\Delta 2$   $n = 26$ , WT ( $w^{1118}$ )  $n = 32$ ).

Differences in overall activity were recorded throughout the day. Mutant flies showed decreased activity levels in the morning, evening and night. During the afternoon period (12:00 p.m. – 06:00 p.m.), however, the mutant flies were more active than the wild type (Figure 3.27 A and C).

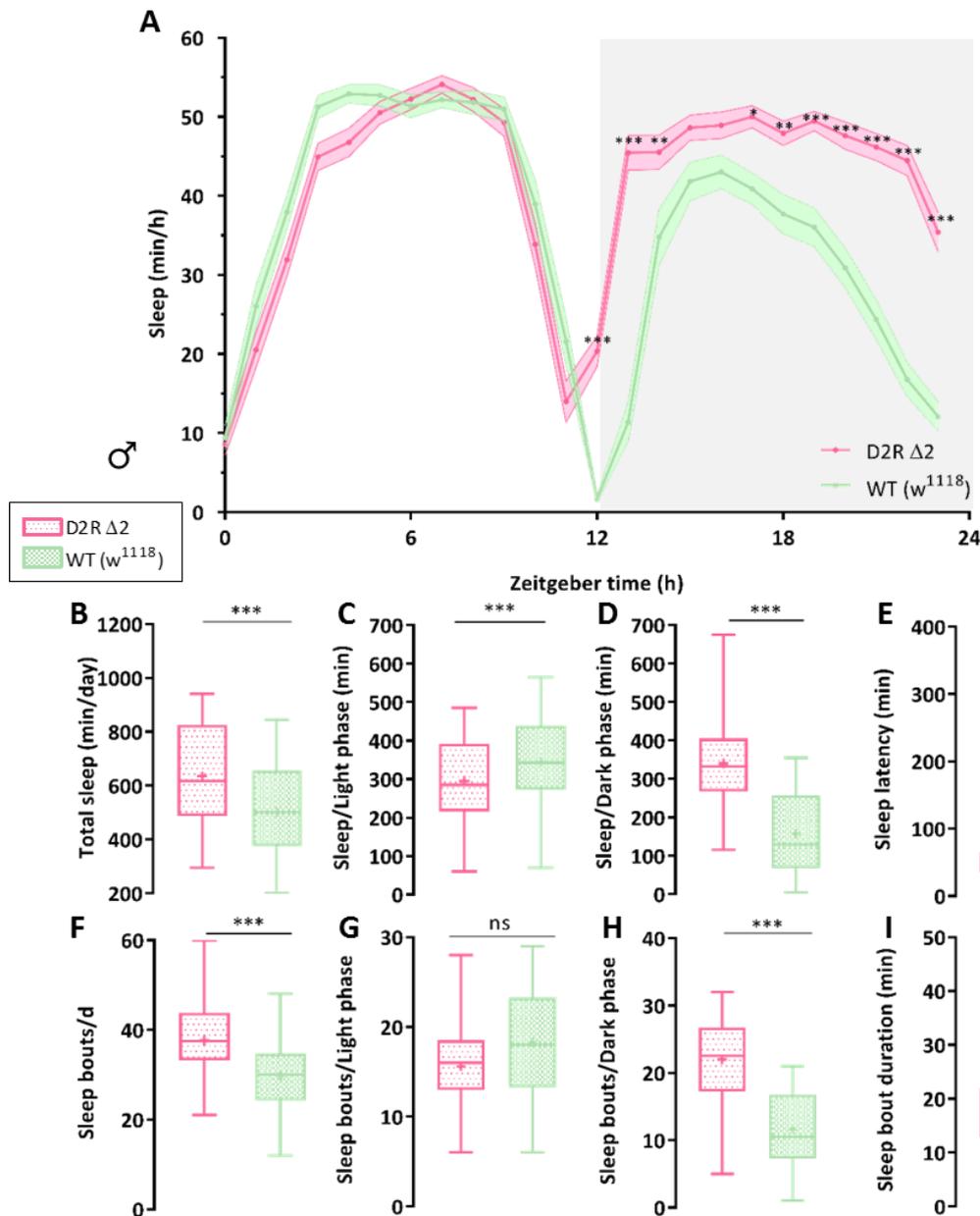
### 3.2.3.2 Total sleep is increased in D2R mutants

As described in the previous section, D2R mutant flies showed decreased levels of total activity. To obtain further insight into the activity pattern, sleep parameters were analysed to examine a potential role of the D2R in sleep behaviour in the fly and to test whether altered sleep levels might account for the reduction in total activity.

In accordance with reduced total activity, the total sleep time was increased by 27% in male D2R mutants (Figure 3.28 A and B) (unpaired t test, \*\* $p = 0.0053$ ; D2R  $\Delta 2$   $n = 28$ , WT ( $w^{1118}$ )  $n = 32$ ). Moreover, sleep is more frequently initiated in knockout flies (Figure 3.28.F) (unpaired t test, \*\*\* $p = 0.0007$ ) but the mean bout length was found comparable to that of wild type control flies (Figure 3.28 I) (Mann Whitney test,  $p = 0.9645$ ).

The time-resolved analysis of sleep of D2R mutant males revealed that the increase in sleep time and bouts takes place during the night (dark) phase (Zeitgeber time 12 – 24) (Figure 3.28

A, D and H), whereas sleep was decreased during daytime (Figure 3.28 A, C and G). Furthermore, after the onset of night (corresponds to environmental cue “light off”), mutant flies quickly entered their first sleep bout after 56.61 min ( $\pm 30.06$  sd) compared to 150.2 min ( $\pm 70.46$  sd) in wild type flies. Thus, sleep latency appeared strongly reduced (Figure 3.28 E) (Mann Whitney test, \*\*\* $p < 0.0001$ ).



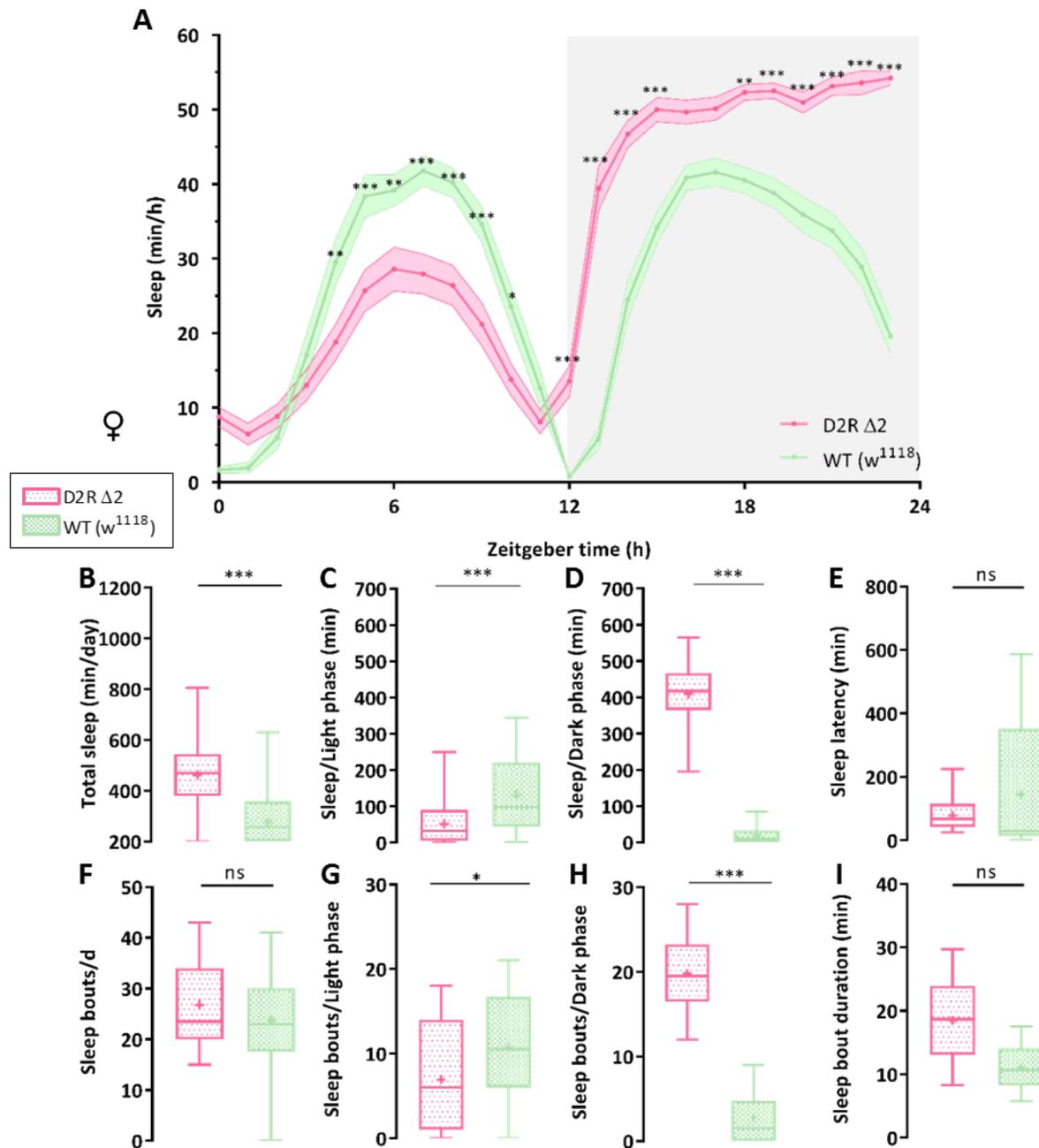
**Figure 3.28 | Sleep behaviour of D2R mutant males.** Activity counts of individual flies were recorded with the DAM system (TriKinetics) at 12:12 light-dark cycle and 25°C (day)/20°C (night) on glucose-agar medium. The circadian sleep pattern is displayed in (A) Two-way ANOVA, followed by Bonferroni multiple comparison test was used to test for significances. The total sleep time (B, unpaired t test,  $^{***}p = 0.0053$ ) and number of sleep bouts per day (F, unpaired t test,  $^{***}p = 0.0007$ ) were increased, what could be assigned to intensified nighttime sleep (D, Mann Whitney test,  $^{***}p < 0.0001$ ; H, unpaired t test,  $^{***}p < 0.0001$ ). The sleep latency was strongly reduced (E, Mann Whitney test,  $^{***}p < 0.0001$ ). The average sleep bout duration throughout the day appeared unaffected (I, Mann Whitney test,  $p = 0.9645$ ), as well as the daytime sleep (C and G, unpaired t test,  $p = 0.0.955$  and  $0.0834$  respectively). (A) Data points represent means are plotted  $\pm$ SEM. Grey shaded background indicates light-off period. (B – I) Plots illustrate the 25% to 75% percentiles (box), median (horizontal line), mean (plus) and min and max values (whiskers). D2R  $\Delta 2$   $n = 28$ , wild type  $n = 32$ . \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.0001$ .

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As a matching characteristic in the sleep phenotype of D2R mutant males and females, the overall time that flies slept during the day was increased. In other parameters, however, slight differences occurred between the sexes.

As mentioned before, D2R mutant females slept by 68% more than the wild type control (Figure 3.29 B, unpaired t test, \*\*\* $p < 0.0001$ ; D2R  $\Delta 2$   $n = 26$ , WT ( $w^{1118}$ )  $n = 28$ ). However, this increase in sleep time arose from extended sleep bout durations (Figure 3.29 I, unpaired t test, \*\*\* $p < 0.0001$ ) and was not caused by a change in the total number of bouts per day (Figure 3.29 F, unpaired t test,  $p = 0.3237$ ).

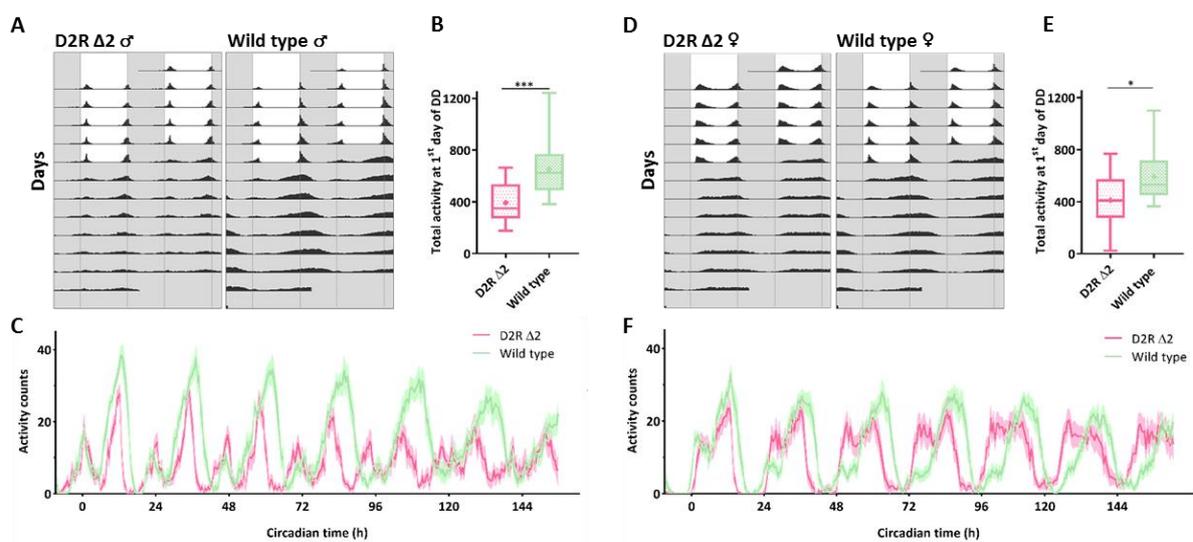
In D2R mutant females, the daytime minutes of sleep and number of sleep phases were less than in wild type flies (Figure 3.29 C and G, Mann Whitney test, \*\* $p = 0.0016$  and \* $p = 0.0255$ ). Nevertheless, during the night, this pattern was reversed and mutant females slept longer in total and more often (Figure 3.29 D and H, Mann Whitney test, \*\*\* $p < 0.0001$ ). In contrast to the observation in male mutants, sleep latency was unaffected in females (Figure 3.29 E).



**Figure 3.29 | Sleep behaviour of D2R mutant females.** Activity counts of individual flies were recorded with the DAM system (TriKinetics) at 12:12 light-dark cycle and 25°C (day)/20°C (night) on glucose-agar medium. The circadian sleep pattern is displayed in (A). Two-way ANOVA, followed by Bonferroni multiple comparison test was used to test for significances. The total sleep time was increased (B, unpaired t test, \*\*\* $p < 0.0001$ ), which was attributable to prolonged sleep bout durations (I, unpaired t test, \*\*\* $p < 0.0001$ ). The number of sleep bouts per day was unchanged (F, unpaired t test, ns), although the flies showed less sleep bouts during the day (G), accompanied by decreased sleep time (C), and a rise in the number of sleep bouts and sleep time during the night (D and H). Sleep latency appeared unchanged (E). (A) Data points represent means  $\pm$  SEM. Shaded background indicates light-off period. (B – I) Plots illustrate the 25% to 75% percentiles (box), median (horizontal line), mean (plus) and min and max values (whiskers); D2R  $\Delta 2$   $n = 26$ , WT ( $w^{1118}$ )  $n = 28$ . \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.0001$ .

### 3.2.3.3 Circadian rhythmicity is affected by D2R deficiency

To examine a potential effect of the D2R deficiency on the circadian rhythm, I determined the endogenous period of mutants. The flies were first entrained to a 12h:12h light/dark (LD) and 25°C/20°C temperature cycle for 5 consecutive days, followed by a release into a “free-running” period, in which all environmental cues to the clock system (temperature and light) were abandoned. Thus, the activity pattern was recorded under constant darkness (DD) at 20°C for 7 days. A subsequent actogram analysis could not detect abnormalities in the general rhythmicity of locomotor behaviour but in the free-running periodicity (Figure 3.30).



**Figure 3.30 | Actograms of D2R mutants.** Locomotor activity of individual flies was recorded with the DAM system (TriKinetics). Flies were entrained to 12:12 light-dark cycle (25°C (day)/20°C (night)) for 5 consecutive days and subsequently kept under constant darkness at 20 °C. Actograms represent mean actograms (smoothed) and shaded areas indicate light-off periods. (A) Males as well as (D) female mutants were found to keep a stable rhythm and period under free-running conditions with a minor phase delay (B) and (E) indicate activity sums in constant darkness for males and females respectively (25% to 75% percentiles (box), median (horizontal line), mean (plus) and min and max values (whiskers)). (C) and (F) show mean activity counts in constant darkness over the course of 7 days for males and females respectively. Error bands represent SEM. \*  $p < 0.05$ , \*\*\*  $p < 0.0001$ .

As already indicated by the activity level analysis of D2R mutants, both male and female flies were entrained to the typical crepuscular activity rhythm (Figure 3.30 A for males, D for females) and the pattern was maintained throughout the free-running period.  $\chi^2$  periodogram analysis revealed a free-running period of 24.5 h for mutant males and 25.06 h for corresponding controls with a phase delay of 4 min/d and 61.6 min/d respectively. The free-running period of mutant females was determined as 24.5 h with 4 min phase delay per day and 25.06 h with a phase delay of 63 min/d for wild type females.

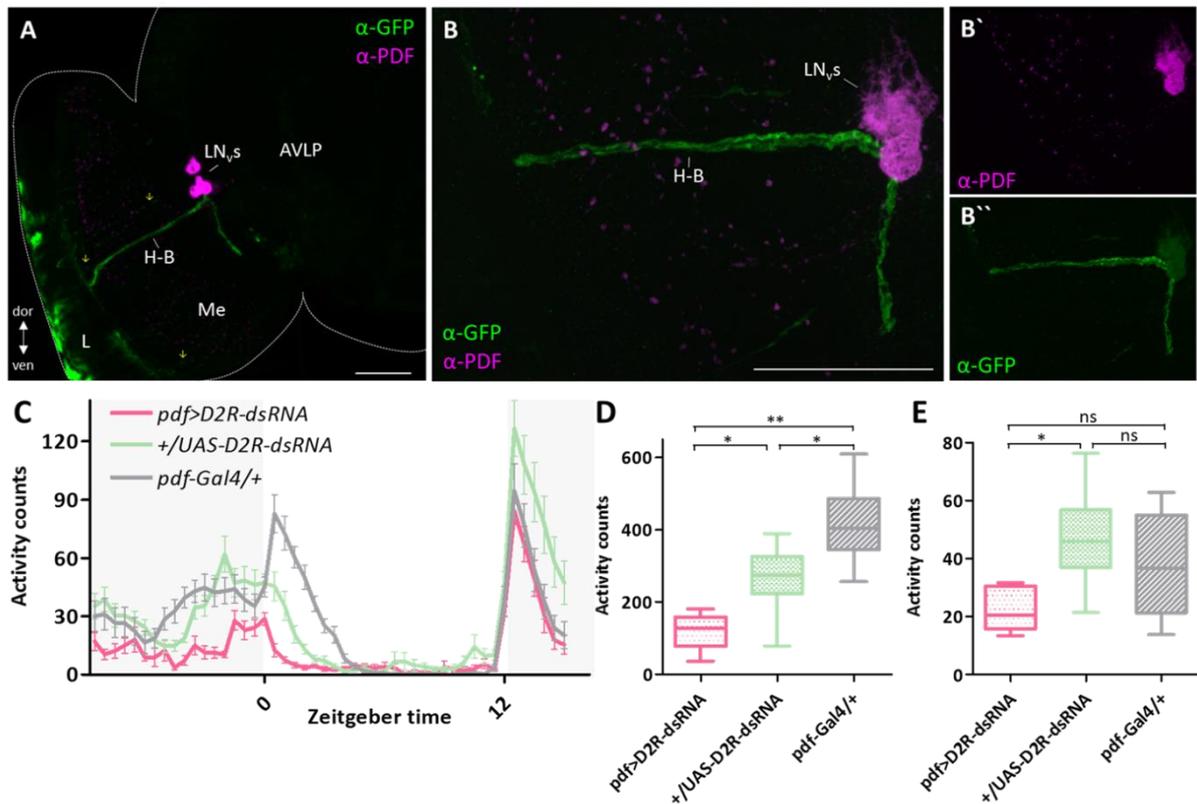
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Furthermore, the total activity was decreased in constant darkness (free-running period, Figure 3.30).

#### 3.2.3.4 Expression of D2R in PDF cells and the Hofbauer-Buchner tract - A potential association with the circadian clock system

As we found the D2R mutant to drastically increase sleep, we were interested in the anatomical/cellular structures underlying this phenotype. PDF expressing cells are known as core clock components and to be involved in sleep regulation (Shang *et al.*, 2011; Sheeba *et al.*, 2008). We thus employed the *d2r*-Gal4 driver line to test for D2R expression in the LN<sub>vs</sub> cluster, which also expresses PDF. GFP signal related to *d2r* promoter activity was detected within the LN<sub>vs</sub>, which were identified by  $\alpha$ -PDF co-staining (Figure 3.31). Moreover, strong GFP signal labelled the Hofbauer-Buchner eyelets. This photoreceptive organ has only recently been described to interact with further photopigments and the LN<sub>vs</sub> to modulate sleep and activity in a light-dependent manner (Schlichting *et al.*, 2016; Veleri *et al.*, 2007).

To test the specific functionality of the D2R in LN<sub>vs</sub> in a first approach, we used RNAi to downregulate the receptor in PDF cells (Figure 3.31 C-E). These flies showed reduced activity levels, however, this result only partially resembled the phenotype of D2R knockouts. The RNAi-mediated knockdown of D2R suppressed both day- and nighttime activity, whereas in mutants only nighttime activity was suppressed and daytime activity increased.



**Figure 3.31 | D2R is expressed in PDF synthesising LN<sub>vs</sub> and the Hofbauer-Buchner eyelet.** A *d2r*-Gal4 line was crossed to the 20xUAS-6xgfp line and brains of progeny were immunohistochemically analysed to visualise the pattern of *d2r* promoter activity (green). Co-staining with  $\alpha$ -PDF antibody (magenta) revealed an overlapping signal, thus indicating *d2r* activity in lateral ventral neurons (LN<sub>vs</sub>) (A and B). Moreover, the Hofbauer-Buchner (H-B) eyelet was strongly GFP positive. Additional *d2r*-related GFP signal was seen in the lamina (L) as well as in photoreceptor cells extending through the medulla (Me). AVLP – anterior ventrolateral protocerebrum. (C) Activity pattern of flies with RNAi-mediated depletion of *d2r* in PDF cells (Mean  $\pm$  SEM; shaded areas indicate light-off phase). The activity levels appeared significantly reduced in these flies as indicated by the sum of activity counts during day (D) and nighttime (E). Plots indicate 25% to 75% percentiles (box), median (horizontal line), and min and max values (whiskers)). *pdf*>D2R-ds-RNA n = 7, w1118/UAS-D2R-ds-RNA n = 8 *pdf*-Gal4/w1118 n = 8. \*  $p < 0.05$ , \*\*  $p < 0.01$ .

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## 4 DISCUSSION

DA is a central messenger molecule implicated in a variety of physiological tasks and behaviours from invertebrates to mammals. In flies as well as in humans, its involvement as neurotransmitter in learning, memory and further cognitive processes, the reward system, endocrine function, modulation of sleep and locomotor control is well known. However, the variety of physiological and behavioural roles of DA requires the sophisticated interplay of underlying molecular elements and correlating cellular/neuronal networks. Only recent methodical advances in biomedical research have led to the recognition of complexity levels and system flexibility in DA signalling in mammals. This established the opportunity to detailed understanding of molecular machineries underlying DA-mediated effects as it is required in the development of effective therapeutic approaches in the treatment of DA-related diseases. Due to the high complexity of the mammalian brain, the use of less complex model systems benefits progress in this field. I have employed the fruit fly *Drosophila melanogaster* as a model to contribute to the understanding of the functions and elements of dopaminergic signalling. The employment of an invertebrate model is favourable as ethical concerns are prevented that might arise in in vivo approaches of certain biological questions. Moreover, the fly's life cycle is short and culturing demands are basic. The usage of genetic tools to manipulate cellular processes is especially appropriate in neurobiological questions as it facilitates the precise understanding of circuit function. In this thesis I examined the functional implications of two *Drosophila* DA receptors, namely DopR and D2R, in a behavioural and metabolic context in the fly. In the following I will discuss the reported results in relation to current questions, research and knowledge.

### 4.1 Potential nonneuronal associations of DopR in metabolism and a sex-specific role in activity modulation

The D<sub>1</sub>-like DA receptor DopR is the best studied DA receptor in the fly, and has been implicated in various physiological tasks, ranging from higher order brain functions (Kim *et al.*, 2007) to direct locomotor control (Pitmon *et al.*, 2016). Besides its function in the central brain, receptor expression was found in peripheral tissues, e.g. salivary glands, fat body, *corpus allatum* and enteroendocrine cells of the gut (El-Kholy, 2010; Gruntenko, Laukhina, and Rauschenbach, 2012; Stephano, 2013), implying non-neuronal functions in general fly physiology. To obtain an overview on the potential physiologic/metabolic relevance of the receptor,

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we have used the DopR knockout mutant *dumb<sup>2</sup>* and investigated life span, metabolic activity and excretion. The life expectancy of *dumb<sup>2</sup>* mutants was increased in males and females, which was paralleled by slightly elevated metabolic activity. Moreover, *dumb<sup>2</sup>* mutant males suffered a reduced excretion rate but faecal spots were bigger in size, which potentially created a balance in the total excreted volume. Although further experiments are needed, the results might indicate DopR involvement in modulation of metabolism/certain physiological traits in general (in this context, please see also “DopR as a potential upstream modulator of the diuretic hormone 44 in the *pars intercerebralis*” (3.1.4)). It should be noted that the *dumb<sup>2</sup>* knockout line does not represent a full knockout but shows abnormal, tissue-specific receptor IR (Kim *et al.*, 2007; Pitmon *et al.*, 2016), which should be considered in the interpretation of results.

We have furthermore employed *dumb<sup>2</sup>* to examine the receptors involvement in activity and sleep modulation. However, a detailed characterisation of the mutant has already been published by Lebestky *et al.* (2009) and Ueno *et al.* (2012). In our hands, *dumb<sup>2</sup>* mutant males were found to be less active concerning the total daily activity, although the strongest reduction was recorded during the afternoon and rather mild activity decrease was recorded for the evening and night. The total activity was unchanged in the morning. Interestingly, the mutants showed the same activity level as controls, indicating no general impairment of locomotor abilities. The decrease in total activity is in accordance with an increase in sleep time. The main increase in sleep was found in daytime sleep; nighttime sleep was only mildly increased. The number of sleep bouts was not affected. Furthermore, sleep latency was decreased in mutants. Since both above-mentioned studies employed male flies, we were interested in the activity and sleep pattern of females. However, *dumb<sup>2</sup>* mutant females were not affected in total activity or sleep.

DA is commonly known to regulate sleep and arousal in the fly and humans and the general statement is that DA promotes wakefulness (Brown, 2016; Liu *et al.*, 2012). For instance, mutants with increased DA levels in the synaptic cleft were found to decrease sleep (e.g. Kume *et al.*, 2005). DopR was shown to hold a regulatory function in two different forms of arousal, that is environmentally evoked arousal as well as sleep-wake arousal. It is well described to mediate the wake-promoting effect of DA in the dorsal fan-shaped body (Liu *et al.*, 2012; Ueno *et al.*, 2012) and is furthermore implicated in the regulation of daytime sleep in the mushroom bodies (Jiang *et al.*, 2016). However, the underlying mechanism for the sex-specific activity phenotype in *dumb<sup>2</sup>* mutants is unclear. Locomotor activity is a general sex-dependent dimorphic trait in *Drosophila*. Differences are thought to arise from sex-specific “tasks”, such as activity phases in males to find females (Helfrich-Förster, 2000). Beyond this ecological function of dimorphism, physiological, e.g. hormonal reasons exist. Imbalances in juvenile hormone during

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development or early adulthood are known to affect locomotor behaviour sex-specifically in older adults (Argue, Yun and Neckameyer, 2013). Moreover, *Wolbachia* infections were reported to affect male activity levels but not females (Vale and Jardine, 2015). It would be interesting to investigate potential hormonal differences in dumb<sup>2</sup> males and females and moreover to look for sex-specific expression and involvement in motor circuits of DopR.

## 4.2 DopR as a potential modulator of Dh44 in the PI

DA is known in mammals to modulate endocrine systems. One of the simplest and best known examples is its inhibiting function of prolactin secretion within the hypothalamus-pituitary association. The hypothalamus-pituitary pathway connects the neuro- and endocrine system in mammals. In insects, the neuroendocrine centre consists of neurosecretory cells located in the dorsal midbrain, encompassing the regions named *pars intercerebralis* (PI) and *lateralis*. These cells send projections to neuroendocrine glands (ring-gland) located at the dorsal tip of the aorta. Based on its structural and functional similarities it is said to be homologous to the mammalian hypothalamus pituitary association (de Velasco *et al.*, 2007). In the analysis of DopR expression pattern by employing the Gal4/UAS system and an enhanced GFP reporter, we have found *dopr*-related GFP signal within neurosecretory cells of the PI, suggesting a DopR-mediated connection of the DA and neuroendocrine system at this site. As a colocalisation of DopR with *Drosophila* insulin-like peptide 2 was excluded in a previous study (Stephano, 2013), we analysed potential overlapping expression with another prominent hormone at this site, which is the diuretic hormone 44 (Dh44). Within the PI, Dh44 is synthesised in 6 specific cells, all of which showed colocalisation with *dopr*-related GFP signal (von Frieling, 2015). Based on high similarity in function and signalling mechanisms, Dh44 is considered to be homologous to the mammalian corticotropin releasing factor (Cabrero *et al.*, 2002; Hector *et al.*, 2009). Indeed, in mammals, DA receptors were reported to exert modulatory effects on corticotropin releasing factor signalling (Bina and Cincotta, 2000). As in the fly brain DA neurons extensively arborize within the PI, Dh44 producing cell bodies were embedded within this network. This indicated dopaminergic synaptic input connections at this site and resembles the situation of clock neurons, which were found to directly contact the Dh44 neurons within this region (Cavanaugh *et al.*, 2014)

To support the presence of functional DopR within Dh44 PI cells, the cAMP reporter *UAS-Epac camps* was employed and changes in FRET signals upon exposure to DA were monitored.

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Individual brains reliably responded to DA application, however, cAMP responses varied in time and intensity. Furthermore, we could show the potential of DA to lower Dh44 protein concentration within PI cells, indicating Dh44 release. Moreover, artificially increased cAMP levels, evoked by expression and activation of the blue light activated AC bPAC, were associated with the tendency of lower Dh44 levels and thus Dh44 release from the PI.

To further test DopR functionality in Dh44 PI cells, we employed an optogenetic tool to manipulate cAMP levels. As it is known for DopR to act via cAMP, we considered the employment and activation of bPAC as imitation of DopR function. Dh44 is a central hormone and implicated in the modulation of various physiological functions. However, life span assays could show that neither a daily 12 hours cAMP-dependent stimulation of Dh44 signalling nor DopR functional suppression evoked phenotypes that affected life span. As the assays were conducted with unlimited resource availability, it is still possible that, in conditions of resource limitation, these flies would be affected in compensatory mechanisms to balance impairments on associated physiological processes (as shown for affected desiccation tolerance of *Dh44>bPAC* flies). Moreover, the results demonstrate the qualification of bPAC in metabolism studies as it induces cAMP generation to a level beyond cytotoxicity (as this most likely would have influenced life expectancy).

We further examined the potential of altered cAMP levels to modulate Dh44 signalling with regard to known Dh44 functions.

As diuretic hormone, Dh44 was described to influence fluid secretion from Malpighian tubules (Cabrero *et al.*, 2002) and, in accordance with this function, to be involved in osmotic stress resistance, as well as desiccation tolerance (Cannell *et al.*, 2016; Hector *et al.*, 2009). Moreover, as part of a post-ingestive nutrient sensor system, it is involved in the modulation of food-intake related physiological processes. Dus *et al.* (2015) have shown the direct influence of Dh44 on gut motility. In light of this, we tested the effect of altered cAMP levels in PI cells on these physiological traits by analysing the graphical characteristics of excreta. Only *Dh44>bPAC* females showed a change in their excretory phenotype with a reduced excretion rate. Assuming a cAMP-induced Dh44 release from PI cells, this result represents the opposite effect to the finding by Dus *et al.* (2015), who demonstrated Dh44-evoked enhanced gut motility and a higher excretion rate. It might be that our assay design was not sensitive enough to detect the elevated excretion rate. As we performed the experiment over 24 hours, it might be that excretion rates were elevated for a certain time at the beginning of blue light exposure. A constantly raised excretion rate over the course of a day would require increased food intake to a degree

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that the flies were potentially not able to compensate. However, we also did not find changes in intestinal transit times, supporting the finding that cAMP stimulation in PI cells did not increase excretion. Furthermore, we did not obtain evidence for altered diuresis at least from the analysis of excreta characteristics. However, *Dh44>bPAC* flies appeared less tolerant to desiccation with the strongest effect in males. This result might indicate a defect in water-retentive mechanisms that facilitate a certain tolerance to desiccation. Insects in nature need to adjust water-loss in retentive mechanisms to keep homeostatic balance in periods without water (Gibbs, Fukuzato and Matzkin, 2003). It would be interesting to investigate the excretory phenotype of *Dh44>bPAC* flies in further detail, as the cAMP effect on diuresis appears inconclusive. In relation to this, it would be very interesting to examine the relevance of Dh44-mediated diuresis in the metamorphosis of the fly. In *Manduca sexta* (moth) the number of Dh44 producing cells increases during metamorphosis and it was postulated that Dh44 underlies the massive water-loss in the transformation of the caterpillar into imago (Cabrero *et al.*, 2002; Veenstra and Hagedorn, 1991). Cannell *et al.* (2016) described the expansion of Dh44 expression in larval stage 3 and the pupal stage of the fly, indicating a central function of Dh44 in the development of holometabolous insects.

Besides its diuretic function, Dh44 has been found to mediate physiological effects evoked by a post-ingestive nutrient sensor located in the Dh44 PI cells (Dus *et al.*, 2015). This nutrient sensor system enables the fly to distinguish between nutritive and non-nutritive sugars after a period of starvation and promotes the ingestion of the nutritive food. Flies impaired in this sensor system preferred a sweeter non-nutritive sugar to less sweet nutritive sugar but control flies behaved vice versa. To test the potential influence of elevated cAMP levels in Dh44 PI cells in this system, a two-choice assay was performed, in which the flies were given the choice between a nutritive and non-nutritive sugar. *Dh44>bPAC* males showed a tendency to resemble the phenotype of flies impaired in the nutrient sensor system but the statistical evaluation of the test did not reveal significance. An influencing effect of cAMP cannot be excluded at this point but increasing the sample size should contribute to a clearer statement.

Furthermore, we examined the effect of cAMP in Dh44 PI cells on fly activity patterns. A previous study identified Dh44 as a circadian clock output factor that acts downstream of clock neurons to signal rhythmicity information to the fly body. Diminished Dh44 signalling as well as continuous activation of Dh44 PI cells disturbed locomotor rhythmicity (Cavanaugh *et al.*, 2014). However, activation of bPAC in Dh44 PI cells did not impair the rhythmicity of activity but increased activity levels. This effect became apparent under long-term and pulse activation of bPAC and might be attributable to efferent connections of the Dh44 PI cells with the fan-

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shaped body of the central complex. By immunohistochemical labelling of Dh44 we visualised Dh44 PI cell arborisations in the medial and dorsal fan-shaped body. This brain region is functionally comparable to the mammalian striatum and generally known to control activity in the fly. In particular, the dorsal fan-shaped body has been associated with arousal (Liu *et al.*, 2012). Moreover, Cavanaugh *et al.* (2014) reported on similar effects of activity enhancement when they activated neurons associated with the central complex region. Nevertheless, they showed that warmth-gated activation of Dh44 PI cells caused a decrease in rhythmicity but no effect on the activity level. However, the innervation of the dorsal fan-shaped body by Dh44 PI neurons, the presence of the Dh44 receptor 1 in this region (Dh44R1; Dus *et al.*, 2015) and the phenotype of cAMP-dependent enhancement of activity might indicate that Dh44 transmits the circadian clock output information to the dorsal fan-shaped body, thereby contributing to the modulation of the arousal state of the fly.

Taken together, we found evidence that DopR evokes cAMP-dependent Dh44 release from PI cells. Functional implications might concern regulatory mechanisms of water-retention and activity (arousal) modulation in the dorsal fan-shaped body. However, further studies are needed to support these findings. In addition, the possibility of associations to the post-ingestive nutrient sensor system and associated gut physiology needs further testing.

### **4.3 D2R function is implicated in the modulation of metabolic traits**

In this section, I examined the metabolic phenotype of D2R knockout flies. The analyses of life expectancy curves has provided a general overview of the severity of D2R deficiency and revealed a higher risk of death at younger age, indicative of impairments in fundamental body functions. This phenotype was accompanied by slightly elevated metabolic activity. As a general reason for death is the lack of a sufficient amount of resources to maintain body homeostasis, we examined the body fat content as a parameter of energy metabolism in D2R deficient flies. Insects store energy in the form of body fat (TAG) and glycogen. Flies deficient of the D2R stored less TAG than corresponding controls although the feeding rate was unimpaired. Moreover, these flies also appeared less tolerant to starvation stress what might indicate the lack of mechanisms to compensate for the reduced availability of body energy in the form of TAG. However, the reduction in body fat content might arise from impairments in a variety of physiological processes. D2R mutants might suffer from increased energy expenditure that potentially arises from increased activity levels that were recorded for mutants (see below) but

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further mechanism are conceivable. As the gastrointestinal tract represents the central nutrient supplier organ, we tested the functional integrity of the gut by the analysis of excreta. No indications were found for an impaired intestinal physiology or function, however, the amount of excreted material (as indicated by the number of faecal spots) appeared reduced in knockout flies. In correlation with an impairment in feeding initiation (affected PER) this might indicate generally reduced food consumption in flies deficient of the D2R although the feeding rate was unchanged.

The D2R is expressed in the CNS as well as in various peripheral tissues, such as the gut, trachea, salivary glands, Malpighian tubules, the fat body and the *corpus allatum* (El-Kholy 2010; Gruntenko *et al.*, 2012b). As we employed a D2R knockout fly line in this experimental series, the receptor function was disrupted universally in all these body tissues and the observed phenotypic effects cannot be assigned to a specific organ or tissue without extended experiments. Moreover, the deletion within the *d2r* gene via the P-elements f02905 and f02891 affected all of the 7 known *d2r* transcripts (Scholz-Kornehl, 2015; Scholz-Kornehl and Schwärzel, 2016).

Kaplan-Meier analyses of survival rates revealed non-proportional hazard ratios over the course of the entire life span. Consistent in both sexes, the curve of D2R mutants can be assigned to type III survival curves that are characterised by lower age-specific survival in the first half of their life. This characteristic curve shape indicates a “bottleneck” situation which implies the presence of adverse factors affecting survival. However, with increasing age, the death rate decreased and normalised to a level comparable to control flies. Due to the universal effect of the gene knockout an interpretation of this finding is not straight forward. As we could demonstrate a reduction in body energy availability in the adult D2R mutant fly, one might expect this decreased resource availability already to be present at earlier developmental stages. As TAG not only represent a source of metabolic energy but also components of essential biomolecules, e.g. cell membrane lipids, the higher death rate in the first half of adulthood might be caused by developmental defects which are compensated with increasing age. Moreover, the higher mortality rates in early life might also be caused by a direct involvement of the D2R in the regulation of development. As Gruntenko *et al.* (2012) have shown, D2R is expressed in the *corpus allatum*. The *corpus allatum* is one major part of the fly’s neuroendocrine centre and the D2R has been implicated to influence juvenile hormone synthesis in this tissue. It was suggested that the receptor, at least in part, underlies the inhibiting effect of DA on juvenile

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hormone synthesis in larvae, thereby preventing premature metamorphosis. A lack of the receptor might cause a premature onset of metamorphosis with associated developmental deficits and health effects.

Furthermore, the reduction in TAG level might arise from different physiological processes. One likely explanation might be a general impairment of fat metabolism or digestion, since the feeding rate was normal in the mutants. As demonstrated in previous studies, D2R is expressed in the gut (Draper *et al.*, 2007; El-Kholy, 2010; Stephano, 2013), salivary glands (own observation, not shown; El-Kholy, 2010) and the fat body (Gruntenko *et al.*, 2012). All these organs might affect the metabolic or digestive processing of food and the contained fat. An impairment in this process caused by the D2R mutation might lead to reduced uptake or storage of dietary fat. However, the cells expressing the D2R in the gut were found most likely to be intestinal stem cells and their daughter cells (enteroblasts) based on the presence of immunoreactivity to the intestinal stem cell marker Delta as well as by cell shape morphology (Stephano, 2013). Thus, in the gut, the D2R might not be directly involved in the digestive function of cells but rather be involved in the modulation of intestinal cell type specificity. It would be interesting to investigate the distribution and/or composition of cell types in the gut of D2R mutants. In mammals, D<sub>2</sub>-like receptors are furthermore found in the intestinal musculature and are crucial modulators of the intestinal transit time. Especially the absence of D2 receptor in mice has been shown to affect gut motility, leading to shorter intestinal transit times by the absence of DA-mediated inhibitory effect on gut motility (Li *et al.*, 2006). However, the intestinal emptying rate (transit time) of D2R knockout flies appeared comparable to controls and the excretion rate was decreased, which represents the opposite effect to the mammalian phenotype. Thus, the involvement of D2R in the modulation of fly gut motility is rather unlikely and the decreased excretion is probably caused by other factors. The function of D2R in the salivary glands is unclear as well. Enzymes excreted with saliva are important at the larval stage, as they dissolve (“digest”) the surrounding medium thereby generating an accessible habitat for fly larvae; they facilitate the digestion of the pupal case and thus adult fly eclosion after completed metamorphosis and are generally considered as part of the digestive tract. The central non-neuronal action of DA in insect salivary gland function is well established and in *Drosophila* an essential role has been demonstrated for the D<sub>1</sub>-like receptor DAMB in the coordination of metamorphosis (Regna *et al.*, 2016; Troppmann *et al.*, 2014). However, a potential function of D2R in salivary gland function relating to TAG digestion remains to be tested. Furthermore, D2R IR was found in fat body cells (Gruntenko *et al.*, 2012), where the receptor might directly be involved in fat metabolism.

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Nevertheless, based on the amount of evidence, the most likely explanation for reduced body fat levels is a reduction in food intake in the mutants. In *Drosophila*, feeding starts with the sensing of stimulatory food components, such as sugars, by sensory neurons of the legs or labellum. The detection of acceptable food components initiates feeding by stimulating the proboscis extension reflex (PER) (Dethier, 1967). A variety of neuromodulator systems is involved in the regulation of feeding to integrate information on metabolic needs and environmental conditions (Pool and Scott, 2014). DA has been described to influence feeding by multiple mechanisms with the potential to act both inhibitory and stimulating. However, one of these DA-dependent modulation mechanisms acts via D2R within the GNG. The modulation of PER initiation could be assigned to one specific TH-positive neuron, the ventral unpaired medial neuron (TH-VUM), which elicits this taste behaviour in response to the sensing of sugar via D2R (in a satiety-state-dependent manner) (Marella *et al.*, 2012). The stimulatory effect of DA on PER in flies vanished in a D2R mutant background, what is comparable to the PER phenotype we have shown for D2R  $\Delta 2$ . It has further been shown that the TH-VUM input neuron is not directly participating in the tasting process but receives input from upstream cellular, potentially directly sugar sensing pathways. However, other circuitry elements are not clear yet but the involvement of D2R in the regulation of feeding initiation might still be of relevance concerning the food intake. With the FLIC system it was only possible to determine feeding rates without indications on the volume of ingested food. It is conceivable that the food volume is reduced in D2R  $\Delta 2$  mutants but not the feeding rate. In general, the role of DA in the regulation of feeding might represent an evolutionarily conserved function. Impairments of the DA system, especially the reward system and D<sub>2</sub>-like receptor function, have been associated with obesity in mammals (Volkow *et al.*, 2008; Volkow, Wang, and Baler, 2011).

#### **4.4 A potential role of D2R in sleep regulation and circadian clock input pathways**

The conserved and central role of DA in the regulation of motor function is well established in mammals and invertebrates. DA controls locomotor behaviour in correlation with arousal-state modulation dependent on a circadian pattern and in accordance with responsiveness demands to environmental stimuli. Nevertheless, functional mechanisms of DA receptors in the modulation of activity and sleep behaviour in general and with respect to environmental cues is largely unknown. Draper *et al.* (2007) reported on a regulatory function of D2R on locomotor activity as they found that RNAi-mediated knockdown of the receptor resulted in reduced activity levels. In our study, we investigated activity patterns of the D2R  $\Delta 2$  knockout fly line (Scholz-

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Kornehl, 2015). Our results indicate a central involvement of D2R in the modulation of sleep levels in l-LN<sub>vs</sub> and, moreover, potential interactions with multiple light receptive input pathways to the circadian clock.

The investigation of overall activity patterns of D2R mutants revealed generally decreased activity levels in both male and female flies and this phenotype was attributed to increased sleep levels. Consistent for both sexes, flies slept less but were more active during the day (light-on period) and slept more during the night (light-off period). Increased total sleep was accompanied by higher numbers of sleep bouts during the night but not caused by increased sleep bout durations. In addition, activity levels in waking periods were decreased, thus demonstrating hypoactivity and general impairment of the mutants (Andretic and Shaw, 2005). Furthermore, actogram based analysis of circadian rhythmicity showed the ability of D2R mutants to entrain to the 12hrs:12hrs light/dark cycle and to stably keep this entrained period under free-running conditions without phase-delay. GFP reporter-based expression analysis showed *d2r* promoter activity in the compound-eye, Hofbauer-Buchner eyelets and l-LN<sub>vs</sub>, all of which represent cellular structures that serve to transmit photic input to the circadian clock neuronal network (Rieger, Stanewsky and Helfrich-Förster, 2003). RNAi-mediated knockdown of the D2R in PDF expressing cells induced reduced activity levels during day and nighttime. This phenotype only partially resembled reduced activity levels of knockout flies, nevertheless supporting a central role of the D2R in activity level regulation in these cells.

DA is described in mammals and invertebrates to exert a wake-promoting effect and to inhibit sleep, which is most likely caused by reducing arousal thresholds. For instance, in the fly, the DA reuptake transporter mutant *fumin* suffers disrupted DA recycling into presynaptic neurons, leading to increased transmitter levels and correlating effects at postsynaptic membranes. In these mutants, postsynaptic partners of DA neurons are constantly activated and the sleep level of these flies was found to be reduced (Kume *et al.*, 2005; Wu *et al.*, 2008). Moreover, *fumin* mutants showed hyperactivity and increased responsiveness to environmental stimuli, suggesting decreased arousal thresholds. In humans, the DA effect in regulation of sleep and arousal becomes apparent in DA-related diseases, such as Parkinson's or schizophrenia, in which patients suffer from sleep disturbances and associated complications. Eventually the wake-promoting effect of the DA system is "exploited" by pharmacological agents, such as caffeine (Andretic *et al.*, 2007; Volkow *et al.*, 2015), methamphetamine (Ares-Santos, Granado and Moratalla, 2013) and cocaine (Kreek, 1996).

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Locomotor behaviour in general is a rather complex trait controlled by multiple converging mechanisms. The dopaminergic modulation of motor control is conserved and well documented for humans and flies (Beninger, 1983; Riemsperger *et al.*, 2011) and the involvement of different receptor subtypes has been described. Dopamine D2 receptors are known in mammals to take part in this behavioural control (Kelly *et al.*, 1998) and in particular lack of D2R in knock-out mice has been associated with increased sleep levels (Qu *et al.*, 2010), implying striking homologies of receptor functions between mammals and invertebrates.

As reported above, an RNAi-mediated approach demonstrated reduced activity levels in flies with broadly diminished D2R levels (Draper *et al.*, 2007). In addition, an early study showed the modulation of locomotion by the D<sub>2</sub>-like receptor agonist Quinpirole, which was directly applied to the VNC of decapitated flies (Andretic and Hirsh, 2000). This finding might indicate a direct involvement of D2R in motor circuits of the VNC. Besides the overall reduced activity levels, which also include sleep phases, we have found the activity level in waking periods (describing activity intensity) to be reduced. This parameter provides information on the physical condition or ability of the fly to move. The reduced level might be attributed to the general reduced fitness we have found in mutants (described under 4.3). However, it might also be explained by receptor functionality loss in mutants in the VNC or other functional regions in the central brain associated with motor control. For example, we detected *d2r*-related GFP signal in association with the protocerebral bridge, which is implicated in walking speed and activity (Poeck *et al.*, 2008).

However, as stated before, reduction in total activity levels are in correlation with increased sleep in D2R mutants. In *Drosophila*, a state of behavioural inactivity (quiescence), which is accompanied by increased arousal thresholds, is defined as sleep. This condition is comparable to the same-named behaviour in mammals and regulated in homeostatic and circadian clock related processes that involve DA as well as octopamine, GABA, serotonin and neurohormones (Crocker *et al.*, 2010; Diego, Jolla and Diego, 2007; Erion *et al.*, 2012; Greenspan *et al.*, 2001; Hendricks, 2000; Parisky *et al.*, 2008; Shaw *et al.*, 2000). Methodically, fly sleep data is easily derived from activity data with 5 minutes of inactivity being considered as sleep “unit” (Andretic and Shaw, 2005). Besides the mushroom bodies and the *pars intercerebralis*, l-LN<sub>vs</sub> are known sleep modulation centres in the fly brain (Crocker *et al.*, 2010; Joiner *et al.*, 2006; Sheeba *et al.*, 2008). l-LN<sub>vs</sub> are part of the circadian clock control circuit, which is represented by ~150 cells in each brain hemisphere, that controls sleep and activity rhythms (Peschel and Helfrich-Förster, 2011). This circuit comprises clock neurons that can be broadly grouped into

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lateral and dorsal neurons. Lateral neurons are further subdivided into large and small ventrolateral neurons and dorsolateral neurons. The ventrolateral neurons are located at the base of the medulla and all but one express pigment-dispersing factor (PDF) (Helfrich-Förster and Homberg, 1993). PDF is a central clock regulator that synchronises clock cells in the absence of light as a clock trigger and furthermore acts to coordinate activity peaks in a day-time-dependent manner. To functionally characterise these cells, it has been suggested that the subgroup of l-LN<sub>vs</sub> transmits light information to the circadian clock circuit, whereas s-LN<sub>vs</sub> act as central oscillators to control the morning and evening peaks of activity (Rieger *et al.*, 2006). Moreover, l-LN<sub>vs</sub> were found to act in the modulation of sleep and arousal, independent of the function of s-LN<sub>vs</sub> (Sheeba *et al.*, 2008). Central modulators of this function in l-LN<sub>vs</sub> are light, octopamine and DA (Shang *et al.*, 2011) and indeed, previous studies indirectly pointed to a role of D2R in sleep regulation in these clock neurons (Gmeiner, 2014; Shang *et al.*, 2011). Shang *et al.* (2011) provided evidence for DA signal to exert stronger effects on arousal than octopamine. However, the wake-promoting effect of DA was found to be light-sensitive and suppressed by light, thus illustrating l-LN<sub>vs</sub> as integration centre for light as arousal trigger and endogenous sleep-promoting signals. PPL2 DA neurons were demonstrated to provide direct input to l-LN<sub>vs</sub> and to elicit cAMP generation. Furthermore, experimental activation of DA neurons was only suppressing nighttime but not daytime sleep indicating the inhibitory effect of light on DA-dependent wakefulness. As DopR, DopR2 as well as D2R transcripts could be verified in l-LN<sub>vs</sub> (Kula-Eversole *et al.*, 2010; Shang *et al.*, 2011) this light-mediated inhibition of DA evoked cAMP signals was suggested to be modulated by light induced enhancement/up-regulation of D2R signalling as it represents the sole inhibitory DA receptor in *Drosophila*. Support for this was obtained by increased DA-induced cAMP levels in conditions of D2R depletion. However, the postulated model described above seems to be well supported by our data: D2R  $\Delta 2$  mutants were found (1) to sleep less during the light phase and (2) to increase sleep during the night. As in the mutants the inhibition of the wake-promoting DA effect is absent, it appears as a consequence that the flies sleep less during the day. The increased sleep during the night phase might represent a sleep rebound to compensate the increased daytime activity (Cirelli and Bushey 2008) as it is known for l-LN<sub>vs</sub> to mediate the wake-promoting effect only during the light phase under normal physiological conditions (Shang *et al.*, 2011). Nevertheless, it might be that additional compensatory mechanisms influence this signalling node in mutants. In fact, further aspects have to be considered concerning the role of D2R in sleep regulation. As stated above, we detected *d2r*-associated GFP signals in in two of the 3 photoreceptive organs in the fly, namely the compound eyes and the Hofbauer-Buchner eyelets.

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Furthermore, the photopigment Cryptochrome is present in l-LN<sub>vs</sub> itself. The potential of these photoreceptive organs as well as the photopigment to provide input to the circadian clock and to modulate locomotor activity was demonstrated by Rieger *et al* (2003). Thus, all of these light input pathways to the circadian clock might be influenced by D2R and underlie or contribute to the observed sleep phenotype of mutants. Cryptochrome, for example, is the photopigment associated with DA nighttime activity stimulation directly in l-LN<sub>vs</sub> (Kumar, Chen and Sehgal, 2012). The Hofbauer-Buchner eyelets were recently found to act against the effects of Cryptochrome and the light information that is mediated by the compound eyes particularly in relation to the l-LN<sub>vs</sub> (Schlichting *et al.*, 2016). In relation to this, it has to be noted that the mutant appeared very stable in its rhythmicity as derived from the actogram analysis. Again, Hofbauer-Buchner eyelets were described to mediate entrainment but also phase shifting in free-running periods (Veleri *et al.*, 2007). So far, there are multiple mechanisms known that might contribute to the abnormal sleep pattern of D2R mutants. However, the interaction of cellular and molecular elements is complex in LN<sub>vs</sub> and further experiments are needed to clarify potential D2R implications in this network.

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## CURRICULUM VITAE

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## Publications

### Journal articles

- Fink C, Schukies S, von Frieling J, Roeder T (2014) Molekulare Analyse aminerger Signalwege im ZNS der Taufliege. *BioSpektrum* (6):636-639

### Conference presentations

- Schukies S, Sperstad SV, Leippe M (2013) A potential Toll-like receptor of *Macrostomum lignano*. 7th International *Macrostomum* Meeting, Groningen, the Netherlands.
- Schukies S, Stephano F, Roeder T (2014) Using *Drosophila* to understand the evolutionarily highly conserved dopaminergic neurotransmission. 107th Annual Meeting of the German Zoological Society, Göttingen, Germany.
- Nolte S, von Frieling J, Li Y, Roeder T (2015) Modulation of Dh44 signaling by biogenic amines. 24th European *Drosophila* Research Conference, Heidelberg, Germany.
- Nolte S, von Frieling J, Li Y, Roeder T (2015) Biogenic amines act as modulators of Dh44 signaling in *Drosophila melanogaster*. CSHL Neurobiology of *Drosophila* Conference, New York, USA.
- Nolte S, von Frieling J, Li Y, Selcho M, Wegener C, Roeder T (2016) Biogenic amines modulate Dh44 signalling in *Drosophila melanogaster*. Arthropod NeuroNetwork (ANN), Kiel, Germany.
- Nolte S, von Frieling J, Li Y, Selcho M, Wegener C, Roeder T (2016) Biogenic amines modulate Dh44 signalling in *Drosophila melanogaster*. 109th Annual Meeting of the German Zoological Society, Kiel, Germany.

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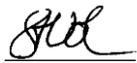
Zuletzt danke ich meiner Familie, die alles so gut mitgemacht hat und mich immer unterstützt, obwohl sie dafür oft selbst zurücksteckt.

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## ERKLÄRUNG

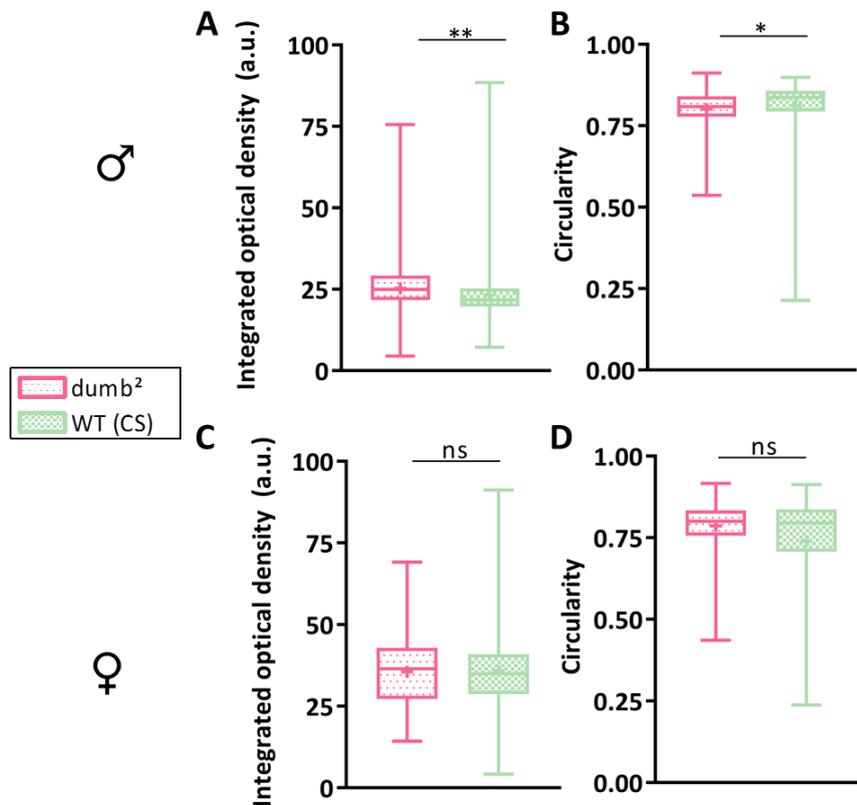
Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft verfasst habe. Neben der Beratung durch meinen wissenschaftlichen Betreuer Prof. Dr. Thomas Roeder wurden keine anderen als die von mir angegebenen Hilfsmittel und Quellen verwendet. Die Arbeit wurde weder in Teilen noch vollständig einer anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt, veröffentlicht oder zur Veröffentlichung eingereicht.

Kiel, den 12.04.2017

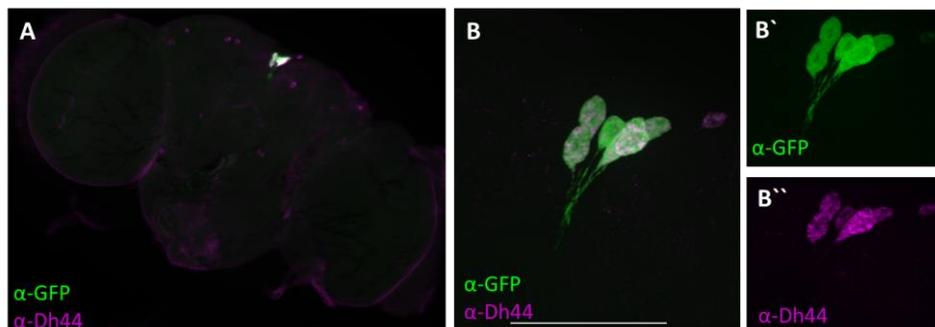


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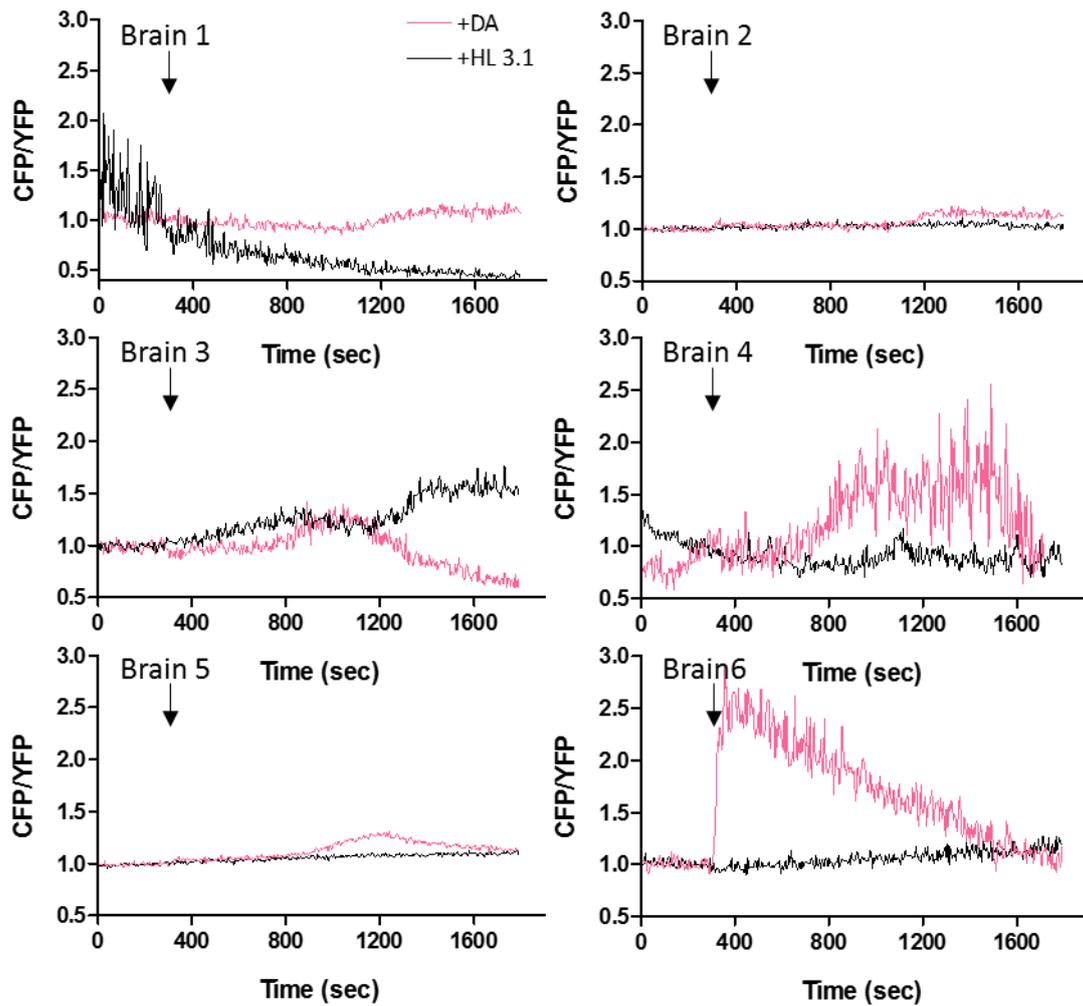
## APPENDIX



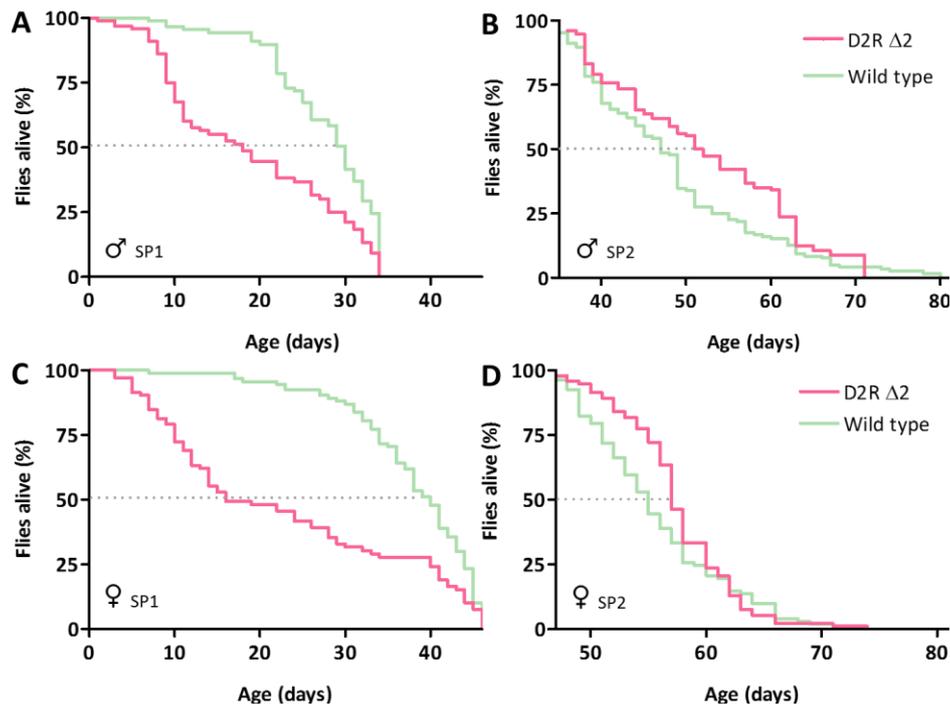
**Appendix Figure 1 | DopR is associated with altered excretion characteristics in males.** To assess the excretory phenotype of DopR mutants, groups of three flies were kept in vials with dyed food and cover slips onto which flies deposited excreta. After 24 hours, the cover slips were analysed by employing a software tool to extract the graphical information of excreta (Wayland *et al.*, 2014). Male DopR mutants were found with significantly higher integrated optical density (Mann Whitney test \*\*  $p = 0.0015$ ,  $n = 10$  each, A) but less circular faecal spots (Mann Whitney test \*  $p = 0.0335$ , B). However, the parameters were unaffected in females. Plots illustrate the 25% to 75% percentiles (box), median (horizontal line), mean (plus) and min and max values (whiskers). \*\*  $p < 0.01$ , \*\*\*  $p < 0.0001$ .



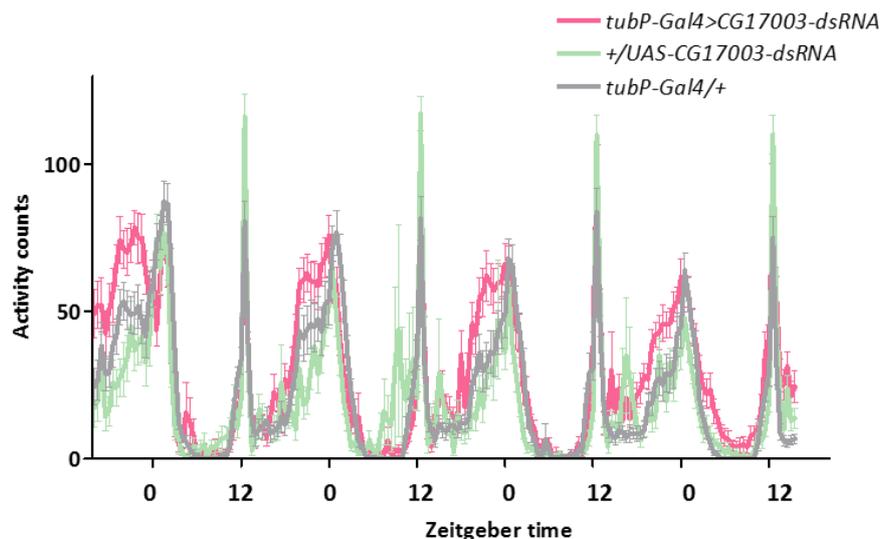
**Appendix Figure 2 | Test for driver line specificity.** Immunohistochemical analysis of GFP reporter signal (green) in *Dh44*<sup>VT</sup>-*Gal4* > *20xUAS-6xgfp* flies.  $\alpha$ -Dh44 containing is shown in magenta. (A) Overview of central brain. (B) Close-up view of PI cells. Scale bars = 50  $\mu$ m.



**Appendix Figure 3 | Dopamine elicits a cAMP response and Dh44 release from PI cells.** To establish DopR function in PI cells, cAMP live cell imaging was performed. Individual brain PI cell responses are illustrated. CFP and YFP fluorescence intensities were recorded from the PI of *dh44 > epac-camps* flies. In the course of the experiments, brains were first exposed to control buffer (HL 3.1) and subsequently to 1 mM DA. Arrow indicates the time point of exposure start. CFP/YFP fluorescence intensities are given in a.u.



**Appendix Figure 4 | Split Life span curves for D2R mutants.** Survival curves of D2R mutants showd non-proportional hazard-ratios with a higher risk of death in younger mutants. The survival curves were split at their crossing points and the two “survival phases” (SP) were analysed separately. (A and B) Male flies. (A, SP1) corresponds to days 1-34 and (B, SP2) to days 35-80 after eclosion. SP1: D2R  $\Delta 2$  median lifespan: 18 d; wild type median lifespan 30 d; log-rank test \*\*\*  $p < 0.0001$ ). SP2: D2R  $\Delta 2$  median lifespan: 51 d; wild type median lifespan 47 d; log-rank test \*  $p = 0.018$ ). (C and D) Female flies. (C, SP1) corresponds to days 1-46 and (D, SP2) to days 47-74 after eclosion. SP1: D2R  $\Delta 2$  median lifespan: 16 d; wild type median lifespan 40 d; log-rank test \*\*\*  $p < 0.0001$ ). SP2: D2R  $\Delta 2$  median lifespan: 57 d; wild type median lifespan 55 d; log-rank



**Appendix Figure 5 | Locomotor activity of flies with an RNAi-mediated knockdown of the gene CG17003.** Since the D2R knockout additionally covers a knockout of the gene CG17003, the gene was ubiquitously depleted (RNAi) and the activity of males was analysed at 30°C. No relevant phenotype was observed.