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„Synaptic Plasticity in the Drosophila olfactory system"

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1 Introduction

1.1 Plasticity

The ability of animals to respond and adapt to environmental changes depends on many factors. One important factor is the brain's ability to build and break down neural connections. This phenomenon of dynamic maintenance of neuronal connectivity can be seen as (neuro)plasticity (Bennett, Diamond, Krech, & Rosenzweig, 1964; Vrensen, & Cardozo, 1981). Synaptic plasticity can have several causes and is based on various mechanisms. Therefore, the concept of metaplasticity has emerged describing the plasticity of synaptic plasticity (Abraham, & Bear, 1996). Neuroplasticity can occur on different levels. Microscopic changes of individual neurons as well as macroscopic changes such as cortical remapping in response to injury can be observed (Pascual-Leone et al., 2011). Many studies have already shown that certain areas of the brain are still changeable in adulthood (Bennett et al., 1964; Rakic, 2002; Sasmita, Kuruvilla, & Ling, 2018). There is also indirect evidence that certain neurons can regenerate in brains of adult humans (Eriksson et al., 1998).

In humans, the proportion of grey matter in the brain as well as the number and modification of the synapses can change (Zatorre, Fields, & Johansen-Berg, 2012). This was shown in people who have learned to juggle. They found structural changes in brain areas involved in motion (Draganski, Gaser, Busch, Schuierer, Bogdahn, & May, 2004). Another observation was made in London taxi drivers. Areas of their brains involved in memory are larger than in control persons (Maguire et al., 2000).

Although some insights into the cellular mechanisms of structural plasticity in the brain have been obtained in the past, little is known about the different molecular signals that mediate the precise temporal and spatial sequence of neuronal interactions in synaptic remodeling (Budnik, et al., 1996; Heisenberg, Heusipp, & Wanke, 1995; Zhong, & Wu, 1991).

Our most understanding of plasticity derives from studies of animal brains, so they play an important role in neurobiology research to understand the causes and mechanisms of it (Halliwell, 2018). This work focuses on synaptic plasticity in the olfactory system of Drosophila. In Drosophila, compared to vertebrates, neuronal activity appears to be less important for the initial wiring of neuronal circuits (Hildebrand, & Shepherd, 1997). However, the fly shows a rich repertoire of behavioral flexibility according to sensory

experience (Heisenberg, Wolf, & Brembs, 2001). In the Drosophila olfactory system, habituation, the simplest form of behavioral learning in chronic sensory stimulation, correlates with defined structural changes in the corresponding synaptic brain region, an odorant-specific increase in the size of olfactory glomeruli (Sachse et al., 2007).

1.2 The Olfactory system of Drosophila

Olfactory perception in Drosophila begins at the *antenna* and *maxillary palps* (Ayer & Carlson, 1992; Stocker, Lienhard, Borst, & Fischbach, 1990; Rajashekhar, & Shamprasad, 2004). There, approximately 1200 olfactory receptor neurons (ORN) express about 50-60 different types of olfactory receptors in the sensilla of Drosophila (Robertson, Warr, & Carlson, 2003; Vosshall, Amrein, Morozov, Rzhetsky, & Axel, R., 1999). At these receptors an incoming olfactory stimulus is converted to action potentials and transmitted via the axons of the olfactory receptor neurons into the antenna lobes of the Drosophila brain (Vosshall, 2000). The antenna lobes are the main center of the olfactory system and process the received signals. They consist of densely packed nerve fibers, called neuropil, arranged in about 50 glomeruli (Couto, Alenius, & Dickson, 2005; Stocker, 1994). Each ORN with the same receptor projects in the same glomerulus and forms synapses with specific projection neurons (PN) and local interneurons (LN; Gao, Yuan, & Chess, 2000). LNs connect the individual glomeruli with each other, which enables spatial and temporal synchronization. They have no axons and branch only in the primary olfactory bulb. Through inhibitory and excitatory modulation of inter- and intraglomerular communication they have a regulatory function (Chou, Spletter, Yaksi, Leong, Wilson, & Luo, 2010). PNs usually innervate a single glomerulus. Their axons extend to the secondary olfactory center, such as the *mushroom bodies* and the *lateral horn* (Marin, Jefferis, Komiyama, Zhu, & Luo, 2002; Stocker et al., 1990). Here, the odor information is further processed and leads to behavioral actions (Brembs, 2009; Gupta, & Stopfer, 2012; McGuire, Le, & Davis, 2001).

1.3 CO_2 perception and the V-glomerulus

In the odor perception of Drosophila, the detection of carbon dioxide (CO_2) plays an important role (Badre, Martin, & Cooper, 2005). On the one hand, CO_2 can be used as a good indicator for the detection of food sources like fermenting fruits (L'héritier, 1948;

Stocker, 1994). On the other hand, it is a main odor component, which causes a strong avoidance behavior (Suh et al., 2004).

CO_2 perception is further processed in the V-glomerulus (Stocker, 1994). The V-glomerulus lies in the most ventral part of the antenna lobe and receives signals from ORNs expressing the receptor Gr21a or Gr63a (Jones, Cayirlioglu, Kadow, & Vosshall, 2007). The type of activity (stimulation or inhibition) of ORNs provides information of the odorants such as identity, concentration or source (Strutz et al., 2014). The axons of these ORNs form synapses with different PNs and LNs in the V-glomerulus (Couto, Alenius, & Dickson, 2005; Gao, Yuan, & Chess, 2000). Depending on various factors of the perceived odor information, certain neurons are activated. For example, PN-v1 fires at low CO_2 concentrations, while PN-v2 and PN-v3 are activated at high CO_2 concentrations (Lin, Chu, Fu, Dickson, & Chiang, 2013). PN-v1 is an excitatory neuron and, in contrast to PN-v5, projects its dendrites bilaterally, connecting the V-glomeruli of the two hemispheres (Lin et al., 2013). PN-v5 is a unilaterally projected excitatory cholinergic neuron (Batawi, not published). Tanaka, Endo and Ito (2012) have described a PN as AL-t1PN1. The characteristics of PN-v5 and AL-t1PN1 give rise to the suspicion that it describes the same PN.

1.4 Plasticity in the V-glomerulus

Synaptogenesis in the antenna lobe begins in the late pupal phase and continues in the first days of adult life. Despite the basic stability of the cortical map, Devaud, Acebes, Ramaswami and Ferrús (2003) showed that the size of the V-glomerulus in Drosophila does not remain constant throughout the live of a fly. An increase in volume was observed within the first 12 days. Individual olfactory glomeruli showed specific growth patterns. Previous experiments with Drosophila showed that flies exposed to a single odor for several days developed a stimulus-dependent decrease in glomerulus volume (Devaud, Acebes, Ferrús, 2001). Sachse et al. (2007) further analyzed these volume changes. Thus, Drosophila has an ability to undergo activity-dependent plasticity within a glomerulus. Olfactory learning modulates the physiology of glomerular circuits over short time scales. Certain experience can modify the odor pathway both structurally and functionally (Devaud et al., 2003).

1.5 Methods for measuring plasticity

Various techniques can be used to study brain plasticity including measurement of changes in brain structure, function, and of molecular events (Aoki, & Siekevitz, 1988; Bennett et al., 1964; Draganski et al., 2006). Improvements in these techniques help to understand how the plasticity of the brain is related to learning or how recovery in patients with brain injury can be enhanced (Robertson, & Murre, 1999).

So far, other methods such as volume measurement or calcium determination have been used to study plasticity (Monai et al., 2016; Heisenberg, Heusipp, & Wanke, 1995). Therefore, it is beneficial to establish a new method suitable for the observation and evaluation of synaptic plasticity. GFP reconstitution across synaptic partners (GRASP, see materials and methods) makes it possible to mark formed synapses (Feinberg et al., 2008). By using GRASP, subsequent experiments on synaptic activity can be conducted and evaluated more efficiently. I am using this technique to determine different intensities of GFP signals. This allows a quantitative assessment of synapses between the two GRASP partners and a more targeted research. Ideally even small differences can be identified. Thus, making it possible to look for molecular causes of plasticity.

1.6 Aim of the Study

One aim of these experiments is to investigate whether the GRASP method is a suitable indicator for detecting synaptic plasticity in the Drosophila olfactory system. How useful is it to observe, measure and evaluate synaptic activities? Where are the limits of measurable and significant differences?

Another aim is to further investigate the link between the projection neurons PN-v1 and PN-v5 and to examine more closely the dynamics and the conditions of this connection. Therefore, the study analyzes GFP signals of synaptic activity between these partners after CO_2 stimulation patterns. The parameters are appearance, persistence and disappearance of a GFP signal.

2 Material and Methods

2.1 Model Organism

As a model organism for this study the vinegar fly *Drosophila melanogaster* was chosen. The fly stocks were grown at room temperature in small plastic vials (Ø 2.7 cm × h 8.4 cm) filled with a standard medium (see Table 1). For cross-breeding, male flies and female virgins were placed in new vials located in an incubator at 25 °C. After three to four days, flies were transferred to a new vial to prevent the emerging offspring from crossing with each other. Once the flies have hatched, they were collected twice daily (morning and afternoon) and separated into new vials to ensure that the approximate age can be determined on all flies. Flies were classified and sorted according to the correct genotype and, depending on the experimental approach, either kept in ambient air at room temperature or placed in an incubator with 5% CO_2 (Tritech Research, DigiTherm®) at 25 °C.

Table 1. ***Ingredients of the Drosophila standard medium***

Drosophila standard medium (per liter)		
22 g sugar beet syrup	8 g agar	0.5 ml phosphoric acid
80 g corn flour	10 g soy flour	8.4 ml propionic acid
80 g malt extract	18 g dry yeast	12 ml Nipagin (15%)

2.2 Dissection and staining

Prior to dissecting the brains, flies were immobilized with ice, killed in 96% ethanol and washed several times with phosphate buffered saline (PBS). The dissection was performed in a drop of PBS to avoid dehydration of the brain. The fixing, dyeing and mounting of the samples was carried out based on a standardized protocol and is described in Figure 1. All steps were carried out at room temperature unless otherwise specified. For staining the brain structure the primary antibody N-cadh (DSHB) was used together with Goat anti-Rat IgG (H+L) Alexa Flour® 647 (Life Technologies). The media and solutions used during this work are listed in Table 2.

Fixation, staining and mounting protocol
1. Fix dissected brains in 2% PFA on vertical rotor for 60 minutes
2. Remove PFA and quick-wash brains with PBT
3. Wash sample in PBT for 15 minutes on shaker (4 times)
4. Remove PBT and block samples with goat serum (10% goat serum in 0.3% PBT) for 1 hour
5. Remove goat serum, add primary antibody and incubate sample overnight on shaker at 4°C
6. Remove primary antibody and quick-wash brains with PBT
7. Wash sample in PBT for 15 minutes on shaker (4 times)
8. Remove PBT, add secondary antibody and incubate sample overnight on shaker at 4°C *From this step forward, protect the sample from light by covering it with aluminum foil*
9. Remove secondary antibody and quick-wash brains with PBT
10. Wash sample in PBT for 15 minutes on shaker (4 times)
11. Mount brains on an object slide in a drop of VectaShield®
12. Put on cover slide. Use modeling clay to fix the cover slide

Figure 1. **Protocol for fixation, staining and mounting of the Drosophila brains**

Table 2. ***Ingredients used in the immunohistochemistry protocol***

Phosphate-buffered saline (PBS)	PBS + Triton X-100 (PBT)	2% Paraformaldehyde (PFA)
2 l ddH_2O	1 l PBS	1.1 g PFA
151.94 g NaCl	9 l ddH_2O	5 ml ddH_2O
24.92 g Na_2HPO_4 x 2 H_2O	30 ml Triton X-100	35 µl NaOH
8.28 g KH_2PO_4 x 1 H_2O		
Adjusted to pH 7.6 with NaOH		

2.3 CO_2 stimulation pattern

The stimulus used here was an incubation (Tritech Research, DigiTherm®) adjusted to a CO_2 concentration of 5%. Various CO_2 stimulation patterns have been designed to test the quality of the GRASP method. These patterns differed in duration and timing of 5% CO_2 stimulation and were designed to analyze the onset and offset of the GFP signal. In addition, a repeated 5% CO_2 stimulation was performed to check, if a previous stimulation affects the generation of a signal after a subsequent stimulation. As control group flies were exposed to ambient air (0.08% CO_2). The complete stimulation and dissection scheme is shown in Figure 2.

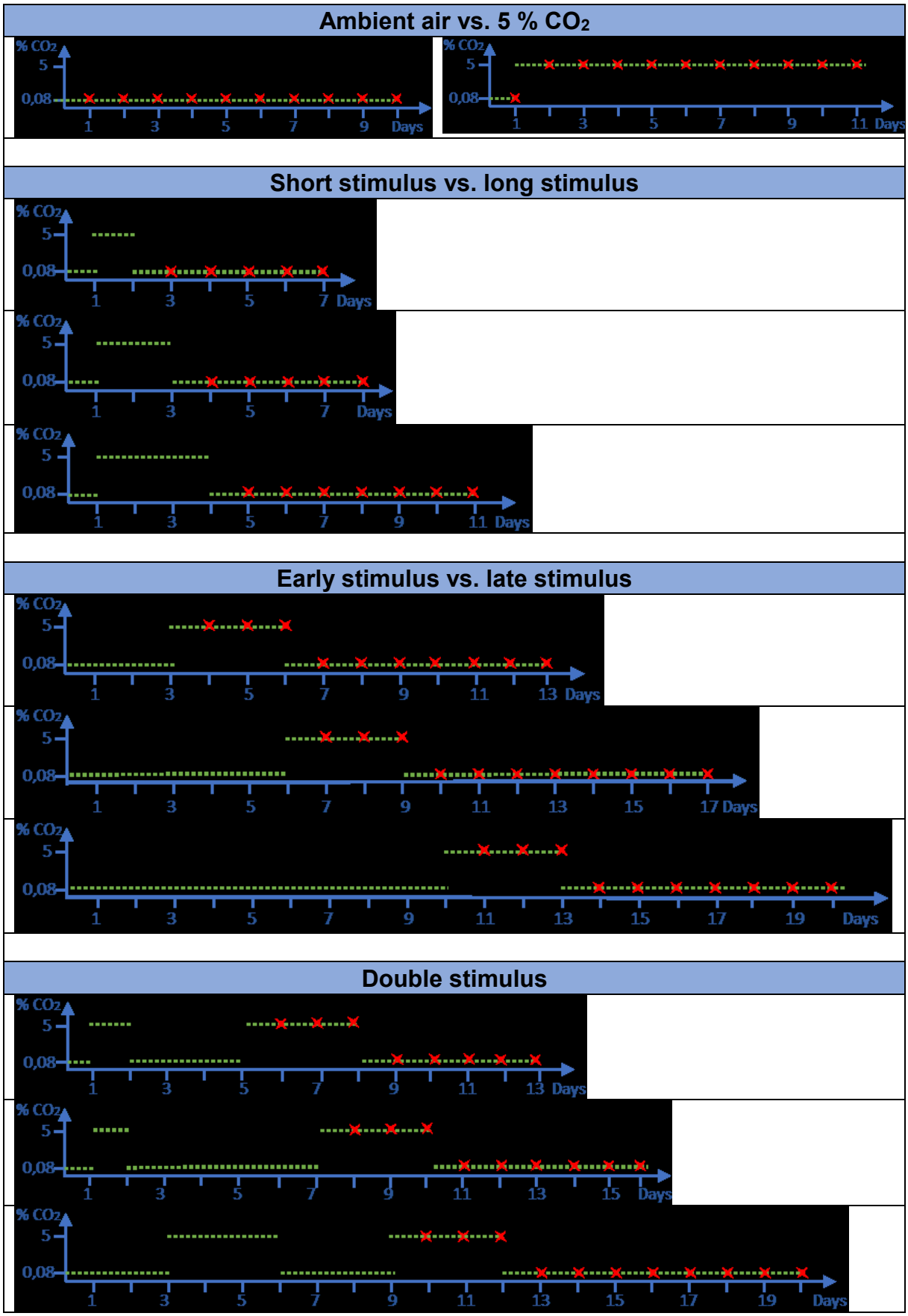

Figure 2. **CO_2 stimulation and dissection scheme.** Flies were incubated with 5% CO_2 or held in ambient air indicated by the green dashed line. The patterns differed in time and duration. The red crosses mark the days the flies were dissected.

2.4 Genetics

2.4.1 Genotypes of the flies

PN-v1, PN-v5 and GRASP lines were used as shown in Figure 3.

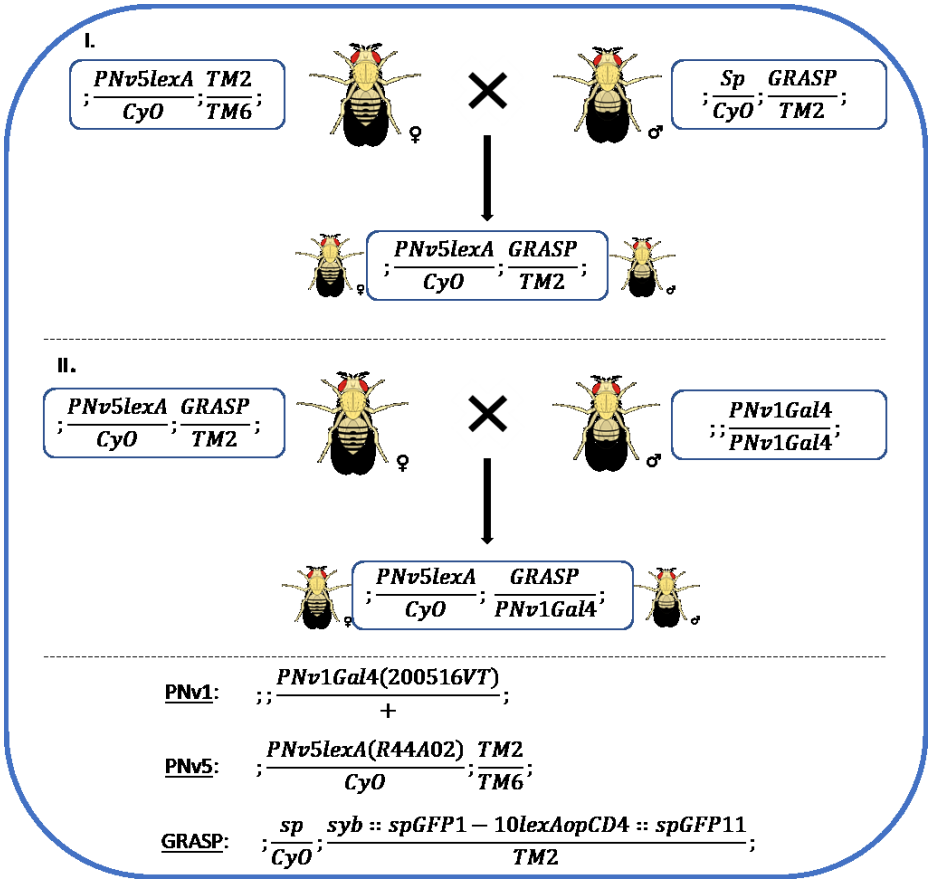

Figure 3. **Genotype crossing scheme.** In the first step PNv5lexA and GRASP are combined. In the second step PN-v1Gal4 is added.

2.4.2 Binary system

A basic genetic tool for these experiments is the binary Gal4/UAS system demonstrated in Figure 4. It allows cell-specific gene expression and consists of two constructs (Kakidani, & Ptashne, 1988; further developed by Brand, & Perrimon, 1993). One part is a cell-specific promoter which directs the expression of the yeast-derived (*Saccharomyces cerevisiae*) transcriptional activator Gal4 encoding gene. The second part regulates a gene of interest through a promoter sequence called upstream activation sequence (UAS; Brand, & Perimon, 1993). To apply this technique, specific male and female fly mutants are crossed. Each sex carries one part of the construct. The offspring's DNA contains both parts, which allows to promote targeted gene expression in specific tissues. The other binary system used in this work is

LexA/LexAop (Lai, & Lee, 2006). It works similarly to the Gal4/UAS system and can be used in parallel creating more possible combinations in spatial and temporal activation of genes of interest in specific neurons.

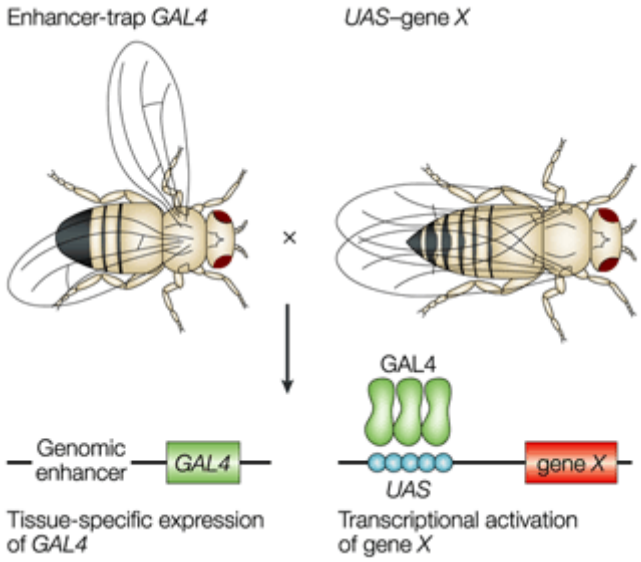

Figure 4. GAL4/AUS binary system in drosophila. GAL4 line and UAS are crossed. GAL4 drive the transcriptionals activation of the gene of interest via UAS (figure from St Johnston, 2002

2.4.3 GFP reconstitution across synaptic partners (GRASP)

An advanced application of the binary Gal4/UAS and LexA/LexAop system is the technique of GRASP (Feinberg et al., 2008). It can be used to visualize synaptic contact between neurons. The GFP is split into two non-fluorescent fragments (Pédelacq, Cabantous, Tran, Terwilliger, & Waldo, 2006). The larger fragment consists of the 1st to 10th beta-strands (GFP^{1-10}), while the smaller fragment is the beta-strand 11 (GFP^{11}) of the GFP molecule (Cabantous, Terwilliger, & Waldo, 2005). The fragments are under the control of Gal4 resp. LexA and thus can be simultaneously expressed in the region of interest. As soon as the two parts are in contact with the respective fragments, the split elements merge resulting in a fluorescent signal (Feinberg et al., 2008). For the experiments here, GFP^{1-10} is bound to neuronal synaptobrevin (syb: $spGFP^{1-10}$), an integral membrane protein expressed in synaptic vesicles (Baumert, Maycox, Navone, De Camilli, & Jahn, 1989) The other GFP^{11} fragment is postsynaptically fused to the transmembrane protein CD4 (Figure 5; Han, Jan, & Jan, 2011). An incoming action potential causes fusion of the vesicles with the presynaptic membrane, which leads to a release of syb:$spGFP^{1-10}$ in the synaptic cleft.

Here it can fuse with the other fragment of the GFP molecule. A resulting green signal indicates that the corresponding synapse has been formed (Macpherson et al., 2015).

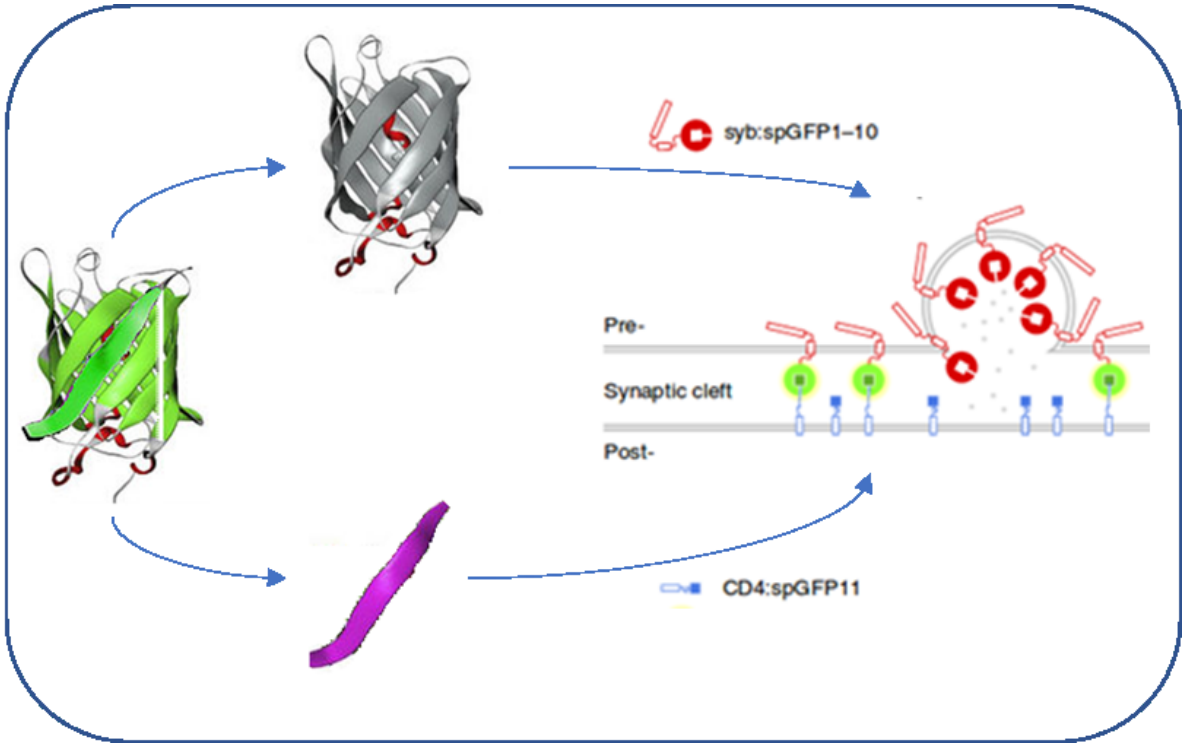

Figure 5. **Scheme of the development of a GFP signal by using GRASP.** GFP is split into two fragments. One fragment is expressed by CD4:spGFP^{11},the other one by syb:spGFP$^{1-10}$. The pre- and postsynaptic expression of the two fragments results in a GFP signal (adapted from Macpherson et a. (2015).

2.5 Image acquisition and data analysis

All Drosophila brains were scanned with a confocal Leica DM6000CM microscope at 20x magnification. The setup was made using the compatible LAS AF software. A HyD laser was used to sample in sections of 1.55 µm with a resolution of 512 x 512 pixels at a rate of 200 Hz. Settings remained unchanged for each scan.

Using the image processing software *Fiji* all images were further processed and analyzed (Schindelin et al., 2012). According to the ROI (Region of Interest) principle the signal intensity measurements were performed (Brinkmann, 2008). First, a circle was created that fits into the region of interest (V-glomerulus). Within this circle, the intensity of the GRASP signal was determined. Since the same circle was used for all scans, a circle size fitting for all brains was chosen. The diameter of the selected circle was 92.8 µm. The layer representing the center of the V-glomerulus was taken out of the scanned images. In the circle, the Fiji built-in program was used with the *Measure* command to determine the intensity of the GFP by comparing the gray levels above a

certain threshold. The circle was placed in three different spots in the V-glomerulus to calculate the mean intensity of the GFP signal. For calculating the background noise, the mean intensity at three adjacent points of the V-glomerulus within the antenna lobe was determined and subtracted from the GFP intensity of the V-glomerulus. This procedure was performed on every measured brain and is demonstrated in Figure 6.

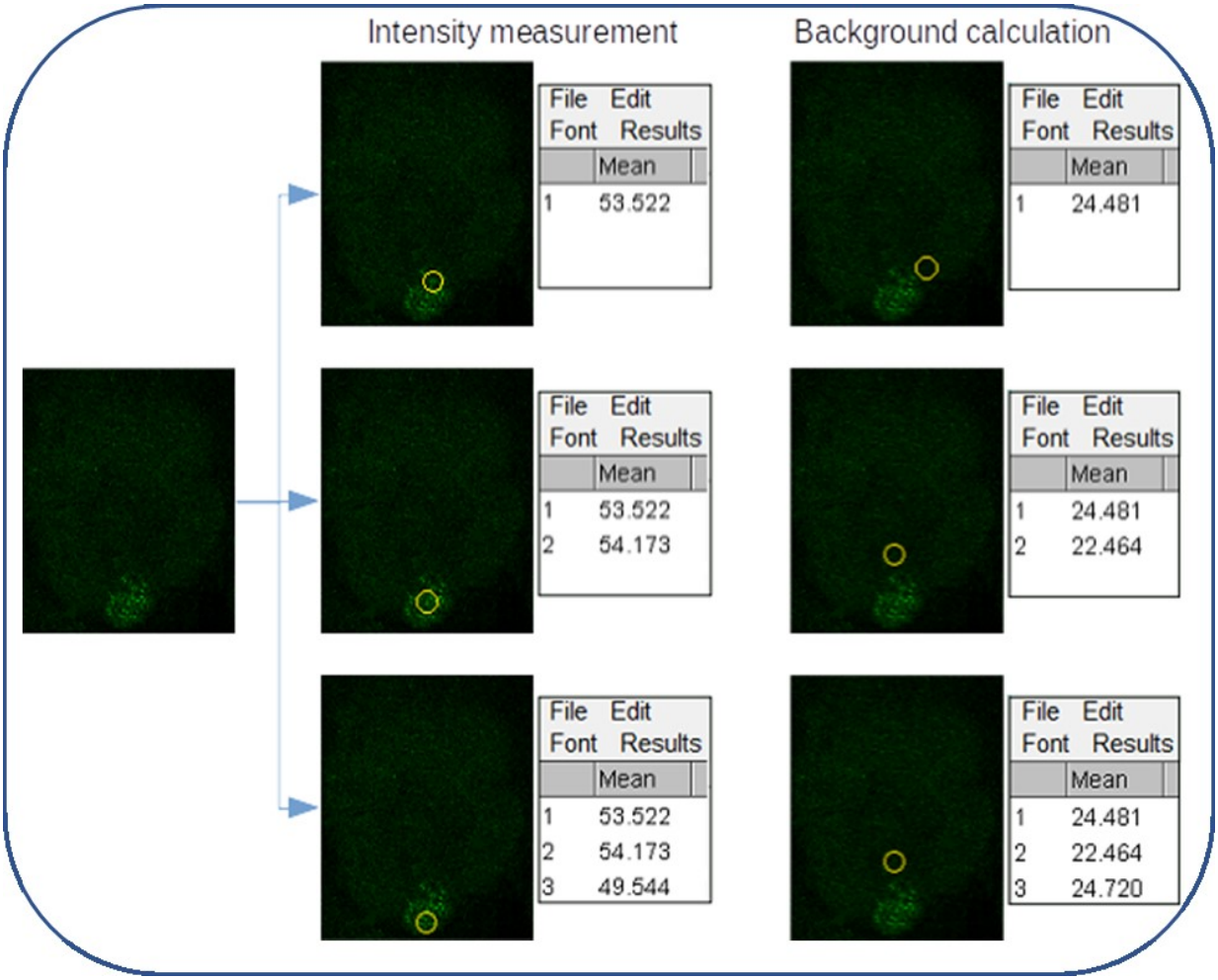

Figure 6. **Intensity Measurement.** The *Fiji* command Measure was used within a circle on three spots in the V-glomerulus and adjacent spots in the Antenna lobe for background calculation and determing GFP signal intensity.

All statistics were performed in SPSS. To detect differences between groups of flies for each day of the experiment an ANOVA was used.

3 Results

This work was used to determine whether the GRASP technique is a suitable method to visualize and measure synaptic plasticity in the Drosophila olfactory system. Genetically modified flies were exposed to pattern of 5% CO_2 concentration. These patterns differed in duration, timing and number of incubations. Subsequently, the GFP intensity in the V-glomerulus was measured.

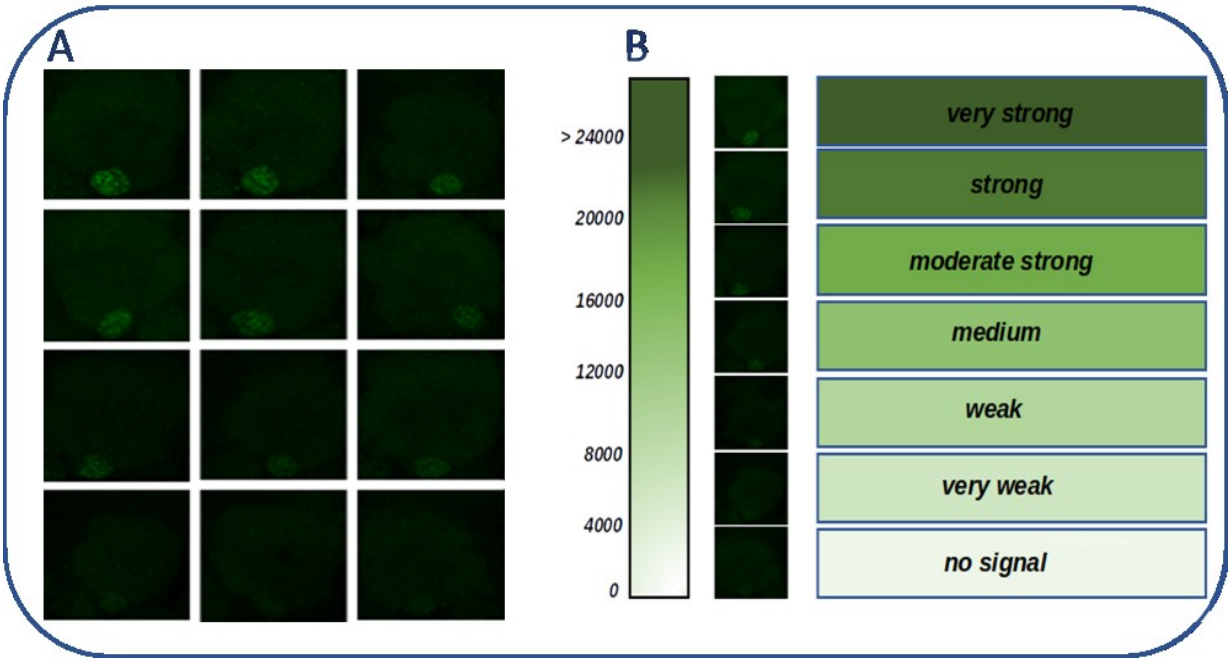

Figure 7. **(A) Examples for the range of the data.** The intensity decreases from upside left to downside right. **(B) Intensity scale categorized according to the measurements.**

The generated data contains a wide range of intensity levels (Figure 7A). To compare the obtained data, a scale has been designed which categorizes the measured GFP intensities. On this scale, areas were defined as being *very strong, strong, moderate strong, medium, weak, very weak* and *no signal* (Figure 7B).

3.1 CO_2 stimulus vs. ambient air

3.1.1 Weak signal on ambient air

Flies exposed to ambient air were dissected for 10 consecutive days and GFP intensity was measured (Figure 8). On average, *very weak* GFP signals were detected on each of the first three days. The mean values of the GFP signal from the fourth to seventh day are *weak*. While an average *very weak* signal was measured after eight and nine days, an average *weak* signal could be measured on the last day. On the 4^{th}, 7^{th} and 10^{th} day, individual brains were measured with *medium* and *moderate strong*

signals. Statistically, the signal intensities of days four (N = 12; p =.038), seven (N = 10; p =.011) and ten (N = 12; p <.01) differ significantly from these of the remaining days. The GFP signals of the remaining days show no significant differences.

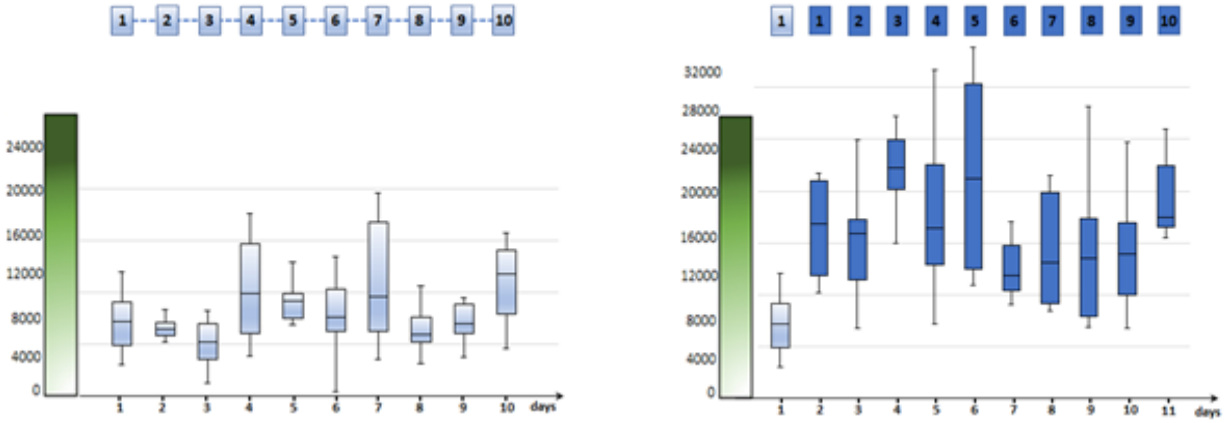

Figure 8. **Intensity measurement under ambient (left) and CO_2 (right) conditions.** Light blue represents ambient air, blue 5% CO_2 incubation. The boxes on top indicate the duration of the respective stimulus.

3.1.2 Strong signal after CO_2 incubation

Flies exposed to ambient air one day after hatching were kept in a 5% CO_2 incubator for up to 10 days (Figure 8). The intensity of the GFP signals on days with elevated CO_2 concentration differs from these on days in ambient air. While *medium* signal levels are observed after one and two days of CO_2 incubation, GFP intensity on the third day of incubation significantly increases to *strong* (N=11, p<.01). After the fourth and fifth day of the CO_2 incubation, a large variation in the signal intensities is observed. Values were detected for both *weak* and *medium* as well as *moderate strong*, *strong* and *very strong*. The average intensity after day four is *medium* and significantly lower than after three days of incubation (N=11, p=.037). After five days of CO_2 incubation, a *strong* signal is measured on average. The brains after the sixth day of CO_2 incubation with an average *medium* signal show significantly lower intensities than after three or five days of incubation ($N_{1,2}$=13, $p_{1,2}$<.01). There are no significant differences compared to one or two days of CO_2 incubation. After ten days of incubation, the average signal strength is *medium*. The associated measurements of this condition are significantly higher than after six, eight, and nine days, but do not differ significantly from the first five days of incubation ($N_{1,2,3}$= 10, p_1< .01, p_2= .019, p_3= .012).

3.2 Onset

3.2.1 Short stimulus vs. long stimulus

In the previous experiment the GFP signal reached the highest level after three to five days of increased CO_2 concentration. It has now been checked whether there is a latency in the generation of this signal. For this purpose, flies were dissected, which, one day after hatching, were exposed to increased CO_2 concentration for one or two days and then kept in ambient air for at least one day (Figure 9).

Flies exposed to higher CO_2 concentration for one day showed a *weak*, *very weak* or *no signal* after several days in ambient air. The GFP signal is significantly lower after the first day in ambient air than after one day exposed to 5% CO_2 concentration (N=10, p<.01). When the CO_2 stimulus lasted two days, a *weak* signal was observed in the subsequent days in ambient air. It was significantly lower than on days with higher CO_2 concentration (N= 14, p<.01). Compared to the control group, flies exposed to ambient air after one or two days of elevated CO_2 concentrations did not show significant differences from flies exposed to ambient air only.

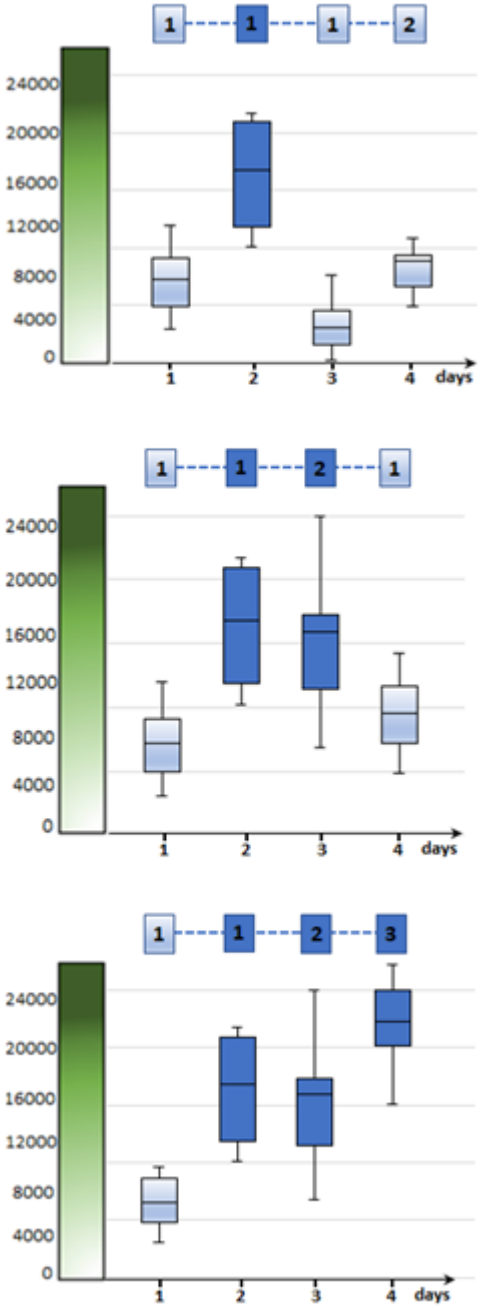

Figure 9. **Intensity measurement with CO_2 stimulus duration of one, two or three days.** Light blue represents ambient air, blue 5% CO_2 incubation. The boxes on top indicate the duration of the respective stimulus.

3.2.2 Early stimulus vs. late stimulus

The next experiment was carried out to check whether the time of exposure to higher CO_2 concentration affects the generation of the GFP signal. Therefore, flies that lived in ambient air for three, six or ten days were exposed to CO_2 for up to three days (Figure 10). The GFP intensity was measured after each day of the CO_2 stimulus.

Flies living in ambient air for three days showed a *very weak* signal on average. The signal increases significantly after one day of higher CO_2 concentration resulting in a *weak* GFP signal (N=12, p<.01). A second day of the stimulus did not cause any further significant changes in the intensity. After three days of higher CO_2 concentration the signal increases significantly to a *medium* signal (N=13, p<.01). Compared to one day old flies exposed to CO_2 the signal is significantly lower after the first day of stimulation (n=12, p<.01) After two and three days of CO_2 incubation, the signal intensities show no differences to flies that were exposed to the stimulus earlier.

The GFP signal in flies exposed to CO_2 six days after hatching shows an increased *weak* signal after one day of CO_2 stimulus (N=14, p<.01). It is similar to the GFP signal of flies living in ambient air for three days before being incubated with CO_2. After two days, there is a significant increase to *very strong* (N=12, p<.01). After three days of CO_2, a *weak* signal is observed on average. The intensity after this stimulation showed no difference to flies that were exposed to the stimulus for one day. It is significantly weaker than that of flies, which were incubated in 5% CO_2 earlier but for the same duration (N=12, p<.01).

On day 10 of ambient air, a *weak* GRASP signal can be observed. Its intensity does not change after one day in CO_2. Another day of incubation leads to a significant increase resulting in a *medium* intensity (N=10, p<.01). A third day with higher CO_2 concentration shows no further significant differences.

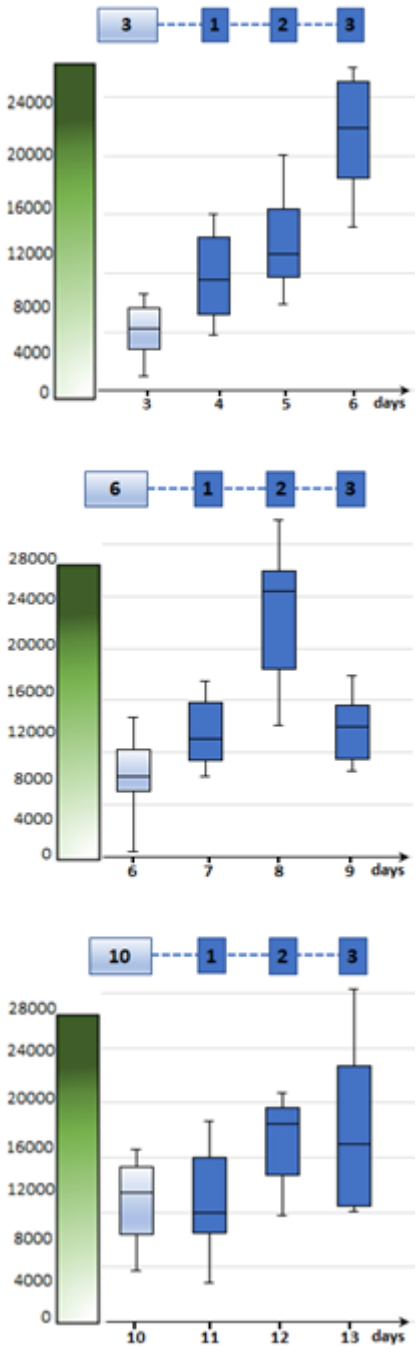

Figure 10. **Intensity measurement with different timepoints of a three-day CO_2 stimulus.** White represents ambient air, blue 5% CO_2 incubation. The boxes on top indicate the duration of the respective stimulus.

3.2.3 Double stimulus

The next experiments investigated whether previous exposure to CO_2 affects the resulting GFP signal after a second CO_2 incubation. The first CO_2 exposure differs in timing and duration and in the time interval to the second CO_2 exposure (Figure 11).

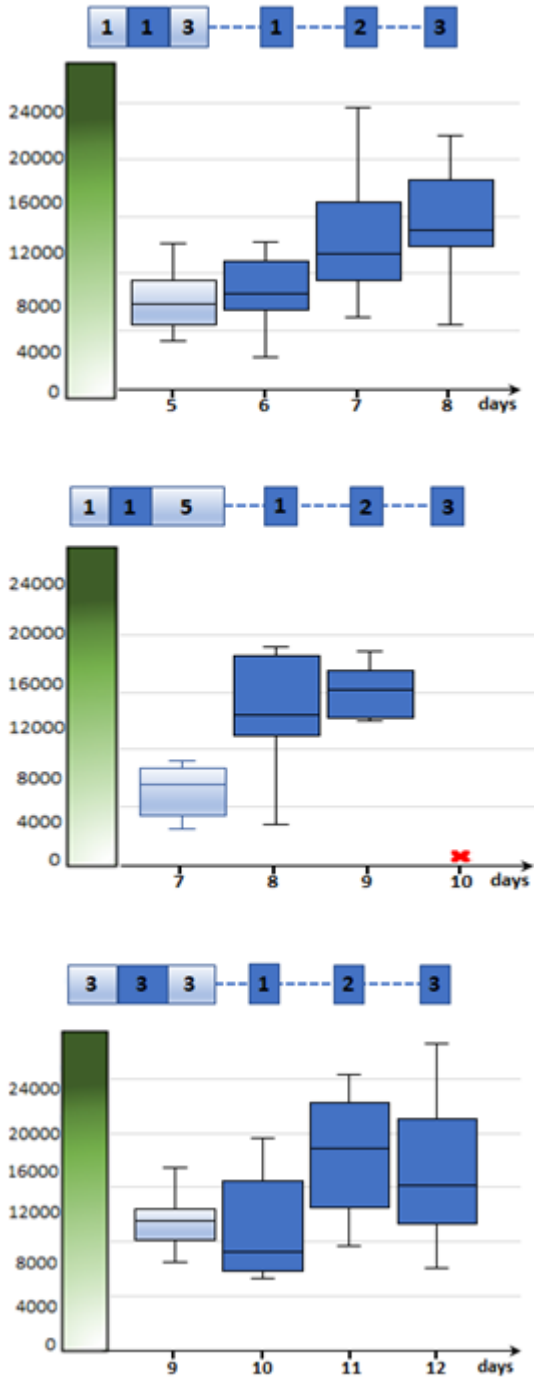

Figure 11. **Intensity measurement after a previous stimulus.** Light blue represents ambient air, blue 5% CO_2 incubation. The boxes on top indicate the duration of the respective stimulus. The red x marks that flies in this condition died.

Flies that received a one-day CO_2 stimulus one day after hatching and then lived in ambient air for three days showed no significant differences in GFP intensity after an additional day with increased CO_2 compared to flies exposed to this stimulus without prior CO_2 experience. The mean intensity is *weak* and does not differ from the previous day in ambient air. After a second day with higher CO_2, the signal increases significantly to *medium* (N=13, $p<.01$). A large variance can be observed resulting in a data set ranging from *weak* to *strong*. The average intensity does not differ from the signal strength of flies without previous CO_2 experience. Another day of CO_2 shows no differences to the previous day. If the stimulus occurs at a similar time, the signal does not differ from those without previous CO_2 experience.

Flies exposed to a second CO_2 stimulus after an interruption of five days showed a *medium* signal after one day of CO_2 incubation. *Very weak* to *moderate strong* intensities could be observed. The signals are significantly different from flies with an ambient air interruption of three days (N=8,

p=.026). After a second day of CO_2 stimulus, no further signal increase and no differences to other conditions can be detected. Flies that have been incubated for three days have not survived.

When the first CO_2 stimulus was prolonged to three days and then followed by another three days in ambient air, a *weak* GFP signal was detected after the first day of the second CO_2 incubation. It is not different from the previous day and does not differ from flies incubated for one day in higher CO_2, regardless of timing of the stimulus or previous CO_2 experience. A second day with CO_2 leads to a significant difference resulting in *medium* and *moderate strong* GFP signals (N=12, p<.01). It is similar in flies exposed to higher CO_2 concentration for two days one day after hatching. Another day of elevated CO_2 shows no changes in the GFP signal. This *medium* signal is significantly lower than in flies incubated in CO_2 for three days, one or three days after hatching (N=8, p<.01).

3.3 Offset

The next experiment examined how the offset of the GFP signal relates to duration, time and previous experience of increased CO_2 concentration. Flies were examined after being exposed to a specific CO_2 stimulus and then lived in ambient air for several days.

3.3.1 Early stimulus vs. late stimulus

One, three, six or ten days after hatching, flies were examined which were exposed to elevated CO_2 concentrations for three days. After up to another seven days of ambient air, the GFP intensity was measured (Figure 12). That of flies exposed to the CO_2 stimulus one day after hatching show different intensities between *medium* and *weak* after living in ambient air for several days. The intensity gets significantly lower (n=10, p<.01) from the first day on in ambient air. The signal strengths in the following days in ambient air differ from each other. The average signal strength is *medium* after one, three, five and seven days. After two, four and six days, brains were observed whose average *weak* GFP intensity was significantly lower ($N_{1,2}$=12, N_3=14, $P_{1,2,3}$<.01). The GFP signals on these days are not different from the ones of the control group.

Exposed to the stimulus three days later, a decrease and a similar pattern of slightly varying signal strengths is seen between *medium* and *weak*. This pattern is less pronounced than in the previously described flies and shows individual significant differences between the days among each other.

If the CO_2 incubation has taken place six days after hatching, a *weak* signal can be observed after one day of ambient air. It shows no differences to the previous day with increased CO_2 concentration. In the next three days, the variance increases and brains with *very weak* to *medium* GFP signals are observed. These changes are not significant. From the fifth day on in ambient air, a significantly lower signal than on the previous days can be seen (N=14, p<.01).

The GFP signals in flies exposed to higher CO_2 after ten days show a similar pattern after subsequent days in ambient air, constant *medium* signals for up to four days after the stimulus and significantly weaker signals from the fifth day on in ambient air (N=12, p<.01).

3.3.2 Double stimulus

Flies exposed twice to increased CO_2 concentration with an interruption of three days and then ambient air were dissected and the GFP intensity was measured

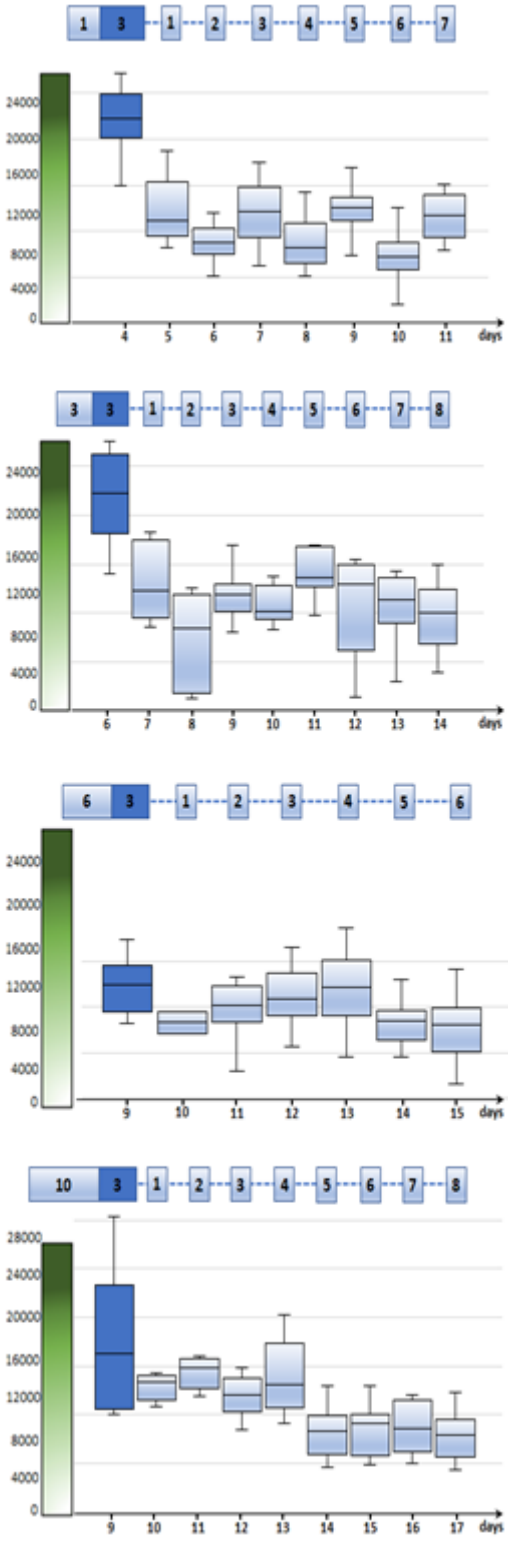

Figure 12. **Intensity measurement of the offset after different timepoints of the CO_2 stimulus.** Light blue represents ambient air, blue 5% CO_2 incubation. The boxes on top indicate the duration of the respective stimulus.

(Figure 13). The first day on ambient air shows no difference to the previous exposure to CO_2. The mean intensity is *medium*. The signal decreases after two to five days in ambient air. The variance is high.

Flies with the same stimulus pattern, but five days in ambient air before the second CO_2 stimulus, show average *weak* and *medium* signals during the following days in ambient air. The variation is high and flies with *strong* signals can be observed.

Flies exposed twice to a three-day stimulus with an interruption of three days show a *weak* signal after one day in ambient air. This signal is significantly lower than on days with higher CO_2 (N=11, p<.01). For the following days in ambient air the signal intensity remains at this level.

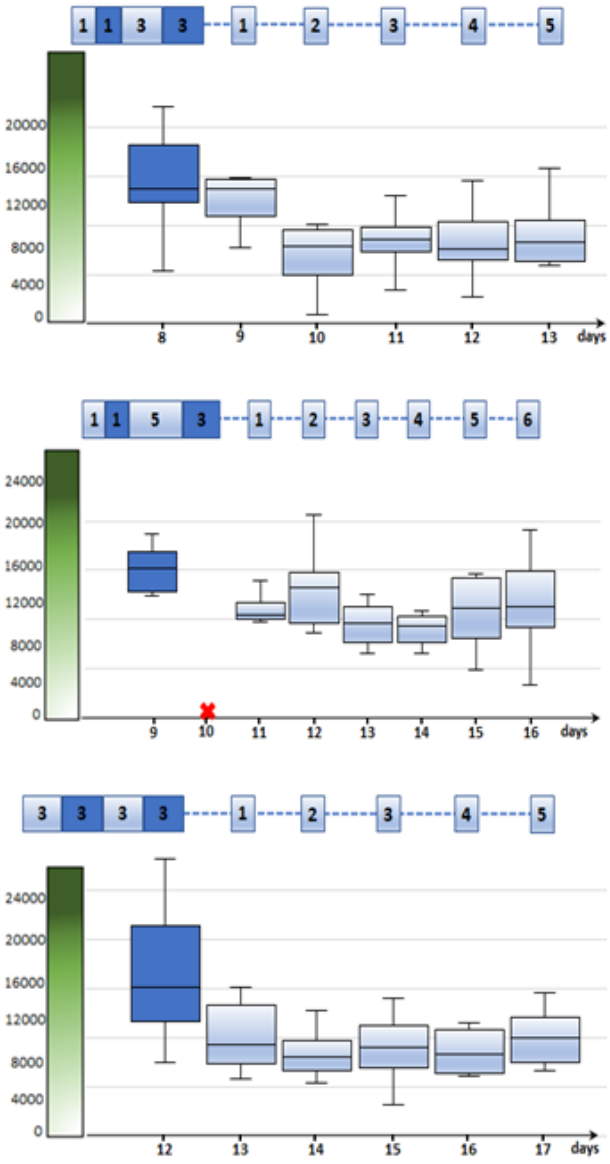

Figure 13. **Intensity measurement of the offset after previous CO_2 experience.** Light blue represents ambient air, blue 5% CO_2 incubation. The boxes on top indicate the duration of the respective stimulus. The red x marks that flies in this condition died.

3.4 CO_2 puff

In the next step, it was checked whether a short puff with pure CO_2 affects the GRASP signal. On the one hand flies were used which lived in ambient air either for one or three days. On the other hand, flies that lived in ambient air for the same time were exposed to the 5% CO_2 concentration for three days. Half of the flies of the respective growing condition receive a burst of pure CO_2 30 minutes before dissecting (Figure 14).

The GFP intensity of flies that lived in ambient air for one day is no different from flies of the same age getting a CO_2 puff. After three days of higher CO_2, a *medium* GFP signal can be measured for both groups. The values show no significant differences.

Flies that lived in ambient air for three days show a *very weak* GFP signal. The mean intensity of the signal is significantly higher when flies receive a CO_2 puff (N=15, p<.01).

When flies are exposed to CO_2 for three days, the signal increases to *strong*. In comparison, flies which received a CO_2 puff, show a *moderate strong* signal on average. The variance is high and there is no significant difference.

4 Discussion

This study investigated whether the GRASP technique can be used as a suitable method for visualization of synaptic plasticity in the olfactory system of Drosophila. For this purpose, the GFP intensity between two types of projection neurons was measured after flies were exposed to specific stimulation patterns of 5% CO_2 concentration. To assess the validity of the GRASP technique and to further investigate the plasticity of this particularly synaptic junction, various CO_2 exposure pattern (variations in duration, time, previous exposure) were performed to examine which parameters affect the onset and offset of the signal.

4.1 Onset

First it was shown how the onset of the GRASP signal is influenced by CO_2 stimulation. For this purpose, GFP intensity was analyzed of flies living in 5% CO_2 conditions compared to control flies living in ambient air.

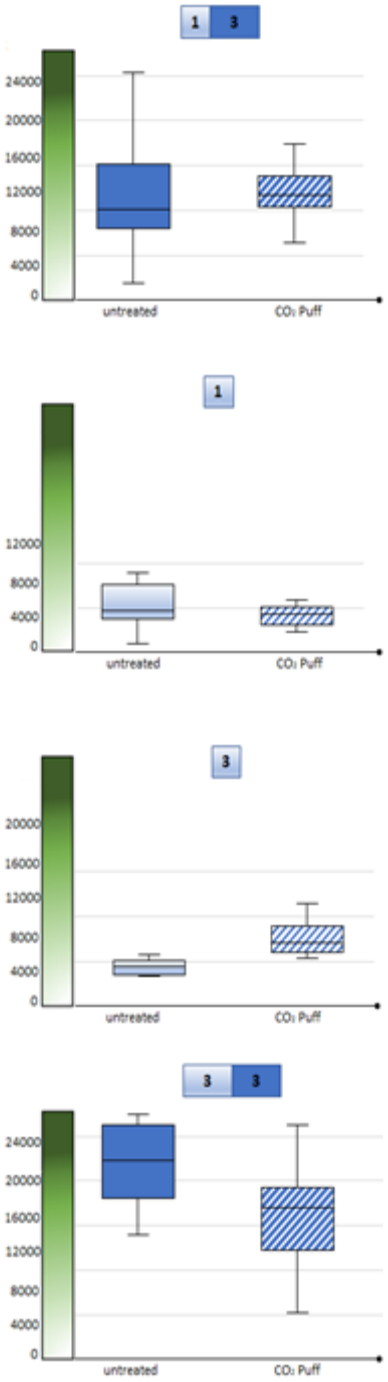

Figure 14. **Intensity measurement with or without a CO_2 puff after different stimulus patterns.** Light blue represents ambient air, blue 5% CO_2 incubation. The boxes on top indicate the duration of the respective stimulus.

4.1.1 Ambient air vs. 5% CO_2 concentration

As it can be seen from the results, there are GFP signals in flies exposed to ambient air only. The existing signal suggests that there are synaptic connections between PN-v1 and PN-v5 under normal atmospheric conditions. The signal intensities are mostly *weak* or *very weak* suggesting only few synaptic connections. But cases of *medium* and *moderate strong* signal intensities indicate that PN-v1 can be strongly linked to PN-v5 even under ambient conditions. Furthermore, such observations may be seen as outliers and therefore need to be considered separately for the assessment of the data. Possible reasons for the occurrence of such outliers will be discussed later.

As soon as flies are exposed to increased CO_2 concentration, stronger GFP signals are measured. This suggests that the perception of increased CO_2 concentration leads to the formation of new synaptic connections between PN-v1 and PN-v5. This provides evidence for an increased GFP reconstitution across synaptic partners (GRASP).

Shown by the data, the average signal intensity is strongest after three days of increased CO_2 concentration. Looking at the absolute values, a maximum of GFP intensity can be observed after five days of CO_2 incubation. This fits to the studies by Sachse et al. (2007) which showed a maximum of volume increase of the V-glomerulus after five days of a CO_2 stimulus. In contrast to the study of Sachse, these experiments showed activity-dependent plasticity after one day of increased CO_2 concentration. This suggests that synaptic changes occur before structural changes. One possible cause is that the increase in synaptic activity causes glial cell formation, which subsequently leads to an increase in volume. However, synaptic and structural changes may have a common cause, but synaptic changes simply occur faster.

4.1.2 Duration of the signal is important for signal increase

The results show that the duration of the stimulus influences the GRASP signal. As mentioned before, one day of exposure to higher CO_2 concentration seems to be sufficient for an increased signal. It has been observed that the signal became less when the flies lived one more day in ambient air. The same was true for flies that were exposed to increased levels of CO_2 for two days. Once the stimulus lasted three days, the GRASP signal was stable for several days. This indicates that synapses can be formed and degraded in the first two days. This high dynamic diminishes after a longer-lasting stimulus and manifests itself in longer-lasting synaptic connections. Sachse et

al. (2007) could show activity-dependent volume changes only after several days of stimulation. It is another indication that structural volume changes take place after this critical window of increased synaptic plasticity.

This early plastic phase of the olfactory system provides behavioral biological benefits assuming the place of hatching is not the location of the later habitat. Thus, this system enables to react very sensitively to environmental stimuli in the early stage after hatching in order to find the most optimal habitat. Once it is found and the existing stimuli persist, the individual can adapt to the characteristics of its new environment.

4.1.3 Timepoint affects onset of the signal

The timing of the stimulus can also influence the genesis of the GRASP signal. The previous results demonstrate that young flies require a stimulus of at least three days for a longer-lasting strong GFP signal. There was no measurable difference in whether the flies were exposed to the stimulus one or three days after hatching. One day with a higher CO_2 concentration leads to a low increase in synaptic activity. It lasts for the next two days and does not continue after five days. Low levels of GRASP were observed in older flies that had not been exposed to higher levels of CO_2. This makes comparison and interpretation of the results difficult because the variance in the measurement of the signal intensity is large. It is possible that synapses have been formed or removed, but this cannot be demonstrated with this data. One reason could be general life-cycle changes in brain structure. This fits to the findings of Devaud et al. (2003), who show that the size of the glomerulus changes during the life of Drosophila. These general volume changes could influence the results because it is unclear whether changes in volume will maintain the number of synaptic connections. Possibly, the larger volume is due to the formation of oligodendrocytes, astrocytes or other glial cells. In this case, as the volume increases, and the number of synapses remains constant, the measured signal intensity of the synaptic density may decrease.

A deviant result was observed in flies exposed to elevated CO_2 concentration for two days six days after hatching. The GFP signal is very strong, but another day with higher CO_2 concentration leads to a significant decrease in GFP signal intensity. Because of unknown reasons, only a few flies have survived in this vial in this approach. This suggests that external circumstances interfered and thus the result was most likely influenced.

The small sample does not allow a valid assessment of the result. In addition, the results show that the olfactory system can produce low GRASP signals in flies exposed to higher CO_2 concentration ten days after hatching. This indicates that new synapses can still be built. The dynamics are lower and the variance of the data larger. A longer-lasting change in the environmental condition is necessary to detect an increase in the GFP signal intensity with this method.

4.1.4 Prior CO_2 exposition has less influence

As another parameter of the generation of a GFP signal, it was tested whether previous exposure to the stimulus influences the generation of the GFP signal after a second phase of 5% CO_2 incubation. Influences on the onset of the GFP signal cannot be found in flies with a previous one-day CO_2 experience. This is reflected in the fact that the same signal intensities were measured in flies without CO_2 experience as in flies with previous CO_2 experience. The observed differences are likely to depend on the timing of the stimulus. This does not exclude that prior stimulus experience could affect the dynamics of the resulting signal, but this method is not sufficient to observe this.

If the stimulus duration or the time between the two phases of increased CO_2 concentration has been prolonged, there is no measurable difference to flies that have received the CO_2 stimulus only once. It should be noted that some dissected flies have reached a relatively high age of up to 12 days under these conditions.

The observable large variation in GFP signal intensities suggests that the experiment may have been exposed to some external influence. One influence can be the setup and the associated procedure of this experiment. The vial must be placed and removed twice in the incubator. Opening and closing the incubator may result in not permanently constant conditions. Special care was taken to ensure that the incubator was opened only for the moment of removal or loading. However, possible effects on the CO_2 concentration in the incubator are not detectable. This existing unavoidable residual risk must be considered interpreting the data.

4.2 Offset

As noted before, at least three days of elevated CO_2 are necessary to build stable synapses. Like the onset of the signal, it was examined how the offset of the GRASP signal is affected by the timing and previous experiences of the CO_2 stimulus.

4.2.1 Young flies show stable signals

The large variance in signal strengths under ambient conditions makes it difficult to interpret the results comparatively. After a one-day recuperation in ambient air, flies which were one day old before CO_2 incubation show stable, but weaker GFP signals. The same was observed in flies which were three days old before CO_2 incubation. This indicates that the synapses still behave plastically in this stage and therefore weaker signals were found even after one day of ambient air. This decrease in signal intensity may also depend on newly formed, unmanifest synapses.

The results show that stable synaptic compounds were formed, which were measured as a *moderate strong* GRASP signal after seven days of ambient air. For three-day-old flies, there is a tendency that from the sixth day on in ambient air the signal becomes uniformly weaker. This tendency is more pronounced in flies that have lived longer in ambient air before CO_2 incubation. The GFP signal increases the first days after increased CO_2 concentration and drops back to the level in ambient conditions from day five on and stays there. This indicates that the system is slower to respond to changes in environmental conditions. The weakening GRASP signal also indicates a decrease in synaptic connections. This decrease is more pronounced in flies exposed to the three-day CO_2 incubation from the 5th day on ten days after hatching. One assumption is that newly formed synaptic connections can be maintained less long in older flies.

4.2.2 Influences of previous experience unclear

For this, flies were exposed twice to CO_2 incubation and then ambient air. There was no significant change and a high variance in GFP signal in flies with two sequences of CO_2 incubation after one day in ambient air. Maybe the formed synapses aren’t a result of the stimulus, but of the lifetime changes in synaptic activity.

Flies in which the previous experience was over three days showed a fast decline in the signal strength. No more significant differences are detected. It is not possible to interpret the data. Possibly, the previous experience has to be longer to detect effects on synaptic activity. In future research, more different experimental conditions are needed.

4.3 CO_2 puff has no impact on GRASP

The data suggests that a single puff of CO_2 has no influence on the GFP signal. In three conditions there is no significant difference in the flies with and without CO_2 puff. Only in one sample after three days of ambient air followed by three days of CO_2, the flies with CO_2 puff had a lower signal than the ones without. This would mean that synapses degraded due to a CO_2 puff. More likely, this experimental sample is an outlier affected by external factors. GRASP is not expressed because of firing of the neurons as a result from the CO_2 puff, but because of the presence of the synapse.

4.4 Possible interfering factors

As seen above, a high variance has often been observed. This can have a variety of causes. Effects of such confounding factors must be controlled in further experiments to verify the validity of the generated data.

Two natural interfering factors can be light and time. The photobleaching known from fluorescence microscopy can influence the GRASP-derived GFP molecule (Song, Hennink, Young, & Tanke, 1995). Exposure to UV light should be avoided to allow GFP analysis as free from external influence as possible. This can be achieved by dissecting in a darkened room.

It might also be considered to skip antibody treatment and mount the brains on a coverslip immediately after fixation. Thus, the brains can be scanned contemporary after opening. This minimizes the risk of the signal being lost due to a possible decline over time.

The deviations were not the same for all experimental conditions. This may have been due to the fact, that those flies forming a respective sample were reared together in a vial. If irregularities have occurred, some vials may have been more or less affected. In addition, flies emit CO_2 depending on environmental conditions, which could temporarily increase the concentration of CO_2 at local sites in the vial (Lehmann, Dickinson, & Staunton, 2000; Williams, Rose, & Bradley, 1997). One way to eliminate this interfering factor is breeding flies individually in a vial. The vials in which the flies lived during the experiments were not changed. This was justified by the fact that unnecessary stress for the flies is avoided. A change during the incubation period was not possible.

Another interfering factor may be the food. It was a standard medium commonly used for Drosophila breeding with a relatively high proportion of yeast which is known for CO_2 production. In retrospect it can be assumed that in food standing at room temperature over a longer period of time, the grown yeast produces a larger amount of CO_2. Thus, the CO_2 content inside the vials may have been different. In subsequent experiments it may be considered to work with yeast-free, CO_2 -free or low- CO_2 food.

To completely avoid the interference factor CO_2, one possible method is to eliminate the CO_2 detection in the flies by targeted mutations. For example, by knocking out or blocking the receptors Gr21a and Gr63a. Alternatively, it is possible to modify them so that they no longer react to CO_2. After such a scenario, no CO_2 perception should take place at all. Even at elevated CO_2 concentration, no GRASP signal should be generated and signal intensities in the V-glomerulus should show no difference in stimulated and unstimulated flies. This would be an additional proof of the specificity of this synaptic connection and of the validity of this visualization method using GRASP.

In order to subsequently use the olfactory system as usual, it would be necessary to replace the CO_2 reception with other physical parameters, such as light or heat. For a light-driven activity, channelrhodopsin-2 (ChR2) or ChR2-XXL could be used (Dawydow et al., 2014; Suh, de Leon, Tanimoto, Fiala, Benzer, & Anderson, 2007). For a temperature-sensitive stimulus transmission, the transient receptor potential (TRP) channels are a possible alternative (Pulver, Pashkovski, Hornstein, Garrity, & Griffith, 2009; Shieh, & Zhu, 1996). In these cases, one would exchange the 5% CO_2 stimulus with blue light or a certain temperature. By this, the stimulus can be set even more targeted and most interfering factors can be avoided

The maximum plasticity can be observed in the first six days. Therefore, it is useful to study this period in more detail in subsequent experiments. In this case, it may be considered to reduce the stimulus interval from 24 hours to 12 hours.

Future research can investigate whether these plastic synaptic activities can be specifically induced or suppressed. This knowledge would be an advancement for science and could provide new opportunities for life and research, not just in medical interest.

4.5 Conclusion

In summary, it was possible to generate a targeted GFP signal in the V-glomerulus between PN-v1 and PN-v5 using the GRASP technique. Furthermore, it was shown that this signal is activity-dependent, as stimulation by increased CO_2 concentration could produce stronger signals. This is indicated by an increased number of synapses between the two neurons. It has also been shown that the development of these synaptic connections depends on the time and duration of the stimulation. There is a critical window, the first three days, in which a particular plasticity in the intensity of the GFP signal could be observed.

Since the variance was sometimes very high, further experiments have to be conducted to confirm the validity of these results. For further experiments, it would be of great benefit to stimulate the olfactory pathway more specifically with the help of (opto)genetic techniques minimizing interfering factors and to carry out even more targeted experiments.

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6 Appendix

6.1 Abstract

The ability of neurons to build and break down neural networks is crucial for the brain to adapt to a variety of environmental conditions. This neuronal plasticity can be observed at the cellular and molecular level. In Drosophila, it has been shown that prolonged exposure to CO_2 causes a reversible volume increase of the CO_2-specific V-glomerulus. The molecular mechanisms behind these structural changes remain largely unknown, due to the lack of suitable methods. In this work, the GRASP (Reconstitution across synaptic partners) method was used to examine whether it is suitable as a valid indicator of synaptic plasticity. With this method, the two projection neurons PNv1 and PNv5 of the V-glomerulus are modified in such a way that GFP is expressed at the synaptic junction of these two neurons. This technique has been combined with defined sensory stimulation patterns to study the dynamics of synaptic plasticity. It was possible to generate an activity-dependent GFP signal between PN-v1 and PN-v5. These signals differed depending on the duration and timing of the stimulation. Moreover, the observed synaptic plasticity matches the temporal dynamics of volume increase in the corresponding glomerulus, including a critical window of maximal plasticity.

6.2 Zusammenfassung

Die Fähigkeit von Neuronen, neuronale Netzwerke auf - und abzubauen, ist für das Gehirn entscheidend, um sich an die verschiedensten Umweltbedingungen anpassen zu können. Diese neuronale Plastizität kann auf zellulärer und molekularer Ebene beobachtet werden. In Drosophila wurde gezeigt, dass eine längere Exposition gegenüber CO_2 eine reversible Volumenzunahme des CO_2-spezifischen V-Glomerulus verursacht. Die molekularen Mechanismen hinter diesen Strukturveränderungen sind aufgrund fehlender geeigneter Methoden weitgehend unbekannt. In dieser Arbeit wird untersucht, ob die GRASP-Methode (Reconstitution across Synaptic Partners) als valider Indikator für synaptische Plastizität herangezogen werden kann. Dazu wurden die beiden Projektionsneuronen PN-v1 und PN-v5 des V-Glomerulus so modifiziert, dass GFP am synaptischen Spalt dieser beiden Neuronen exprimiert wird. Diese Technik wird mit definierten sensorischen Stimulationsmustern kombiniert, um die Dynamik der synaptischen Plastizität zu untersuchen. Es konnte ein aktivitätsabhängiges GFP-Signal zwischen PN-v1 und PN-v5 erzeugt werden. Diese Signale unterschieden sich je nach Dauer und Zeitpunkt der sensorischen Stimulation. Darüber hinaus ist die beobachtete synaptische Plastizität mit der zeitlichen Dynamik der Volumenzunahme im entsprechenden Glomerulus vereinbar, einschließlich eines kritischen Fensters maximaler Plastizität.