Two opposing serotonergic neuronal circuits modulate ethanol preference of *Drosophila melanogaster*

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Contents

Abstract

Decision making is vital for *Drosophila melanogaster* to find food or avoid hazards. When offering wild type flies ethanol enriched food and food without ethanol, flies prefer 5% ethanol containing food (Ogueta et al., 2010). This behavior is caused by olfactory stimuli (Schneider et al., 2012). When the odor information is processed, the decision to approach one odor source has to be converted in movement. In addition, flies tend to climb up the vials after they have been shaken down which is known as negative geotaxis (Kamikouchi et al., 2009). Walking speed measured in negative geotaxis assays can be used to analyze locomotion behavior (Strauss and Heisenberg, 1993). The neurotransmitter serotonin (5-HT) modulates olfactory processing in antennal lobe of *Drosophila* (Dacks et al., 2007). Increased serotonin level by feeding 5-HTP, the serotonin precursor, also causes reduced locomotion activity in flies (Yuan et al., 2006). The role serotonin plays in ethanol preference has not been analyzed. In addition, it is not clear whether serotonin involved in negative geotaxis locomotion.

To dissect the role of serotonin in odor evoked ethanol preference, the function of the key regulator in serotonin signaling–the serotonin transporter (SERT) in olfactory ethanol preference was analyzed. The serotonin transporter removes serotonin from synaptic cleft via reuptake it into the pre-synaptic neuron and therefore terminates the action of serotonin in the synaptic cleft. Even though different *dSERT* mutants have different transcript level, western blot showed that dSERT protein levels are severely reduced in all *dSERT* mutants. The loss of SERT expression is correlated with changes in locomotion since *dSERT16* mutants fail to perform climbing task and also *dSERT18* showed impaired negative geotaxis climbing. *dSERT* mutants were tested for odor evoked ethanol preference. The *dSERT16* mutants could not decide for either food odors or ethanol containing food odor. These results suggested that serotonin is a negative regulator, as increased serotonin levels lead to decreased climbing ability and loss of ethanol odor preference. To confirm the accurate role of serotonin signaling in odor evoked ethanol preference, a dominant-negative version of the serotonin transporter unable to bind serotonin was expressed in different serotonergic neurons in the fly brain to increase serotonin signaling. Expression of this modified transporter in *TPH*-GAL4 driven neurons indeed caused a reduction of ethanol preference. That is due to prolonged 5-HT signaling, since a similar phenotype was observed when flies were fed with the serotonin precursor 5-HTP resulting in increased 5-HT levels (Schläger, 2013). Locomotion did not

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contribute to the reduce preference, since *TPH*-GAL4/UAS-SERTDN flies behaved normal in anti-geotaxis climbing. These results indicate that increased serotonin level suppresses ethanol preference and a subset of serotonergic neurons driven by *TPH*-GAL4 is required for ethanol odor induced behavior. When disturbing dSERT function in *SERT3*-GAL4 dependent serotonergic neurons a decreased preference to ethanol was also recorded. However, these flies exhibit robust ability in climbing. A subset of six serotonergic neurons was found in IP, LP1 and SE1 clusters. Four common serotonergic neurons in IP and LP1 clusters were targeted after compared neuronal expression pattern of *SERT3*-GAL4 with *TPH*-GAL4. Therefore, ethanol preference is modulated by four serotonergic neurons from IP and LP1 clusters in the brain. Surprisingly expression of UAS-SERTDN in *TRH*-GAL4 dependent neurons which covered 83% of serotonergic neurons in CNS does not alter ethanol preference. Beside the same neurons found in *TPH*-GAL4 and *SERT3*-GAL4 drivers, additional serotonergic neurons in CSD, DP and abdominal ganglia were detected. This data suggests another opposing serotonergic neuronal circuit exists to modulate ethanol preference. To verify that preference changes were not due to the strength of different GAL4 expression, UAS-SERTDN was expressed simultaneously in *SERT3*-GAL4 and *TRH*-GAL4 driven neurons. Thereby no change in preference was detected. Same result was observed by expressing UAS-SERTDN in *SERT3*-GAL4/RN2-E-GAL4 driver. However, those flies showed defects in negative geotaxis climbing. RN2-E-GAL4 drives CSD neuron in the brain and a cluster in the abdominal ganglia. Serotonergic cells in CSD cluster and abdominal ganglia are involved in modulating ethanol preference and climbing.

In conclusion, dSERT participates in the modulation of odor evoked preference and negative geotaxis climbing. Serotonin acts as a negative modulator in ethanol preference. Increased serotonin level leads to decreased ethanol preference and four putative serotonergic neurons in IP and LP1 clusters are responsible for this behavior. The preference change is not due to movement ability. Another opposing serotonergic circuit is also involved in regulating ethanol odor evoked ethanol preference in *Drosophila melanogaster*.

Zusammenfassung

Um Futterquellen zu finden oder Gefahren zu meiden sind Entscheidungsprozesse für *Drosophila melanogaster* überlebenswichtig. Wenn wildtypischen Fliegen die Wahl zwischen einer Futter-Duftquelle ohne und mit 5% Ethanol gegeben wird, dann präferieren die Fliegen die Alkohol-haltige Futterquelle (Ogueta et al., 2010). Das Verhalten wird hervorgerufen durch einen olfaktorischen Stimulus (Schneider et al., 2012). Wenn die Geruchsinformationen verarbeitet werden, muss die Entscheidung sich einer Geruchsquelle zu nähern in Bewegung umgesetzt werden. Zudem kann bei Fliegen negative Geotaxis beobachtet werden. Fliegen klettern hierbei nach oben, nachdem sie herunter geklopft wurden (Kamikouchi et al., 2009). Die Laufgeschwindigkeit der Fliegen in einem negativen Geotaxis Experiment dient zur Analyse von Fortbewegungsverhalten (Strauss und Heisenberg, 1993). Der Neurotransmitter Serotonin (5-HT) moduliert Geruchsverarbeitung im Antennallobus von *Drosophila* (Dacks et al., 2007). Durch die Fütterung von 5-HTP (Vorstufe von Serotonin) erhöhte Serotoninspiegel resultieren in eingeschränkter Bewegungsaktivität (Yuan et al., 2006). Die Rolle, die Serotonin in Ethanolpräferenz spielt, wurde bisher noch nicht analysiert. Darüber hinaus ist nicht klar, ob Serotonin an der Fortbewegung bei negativer Geotaxis beteiligt ist.

Um die Rolle von Serotonin in olfaktorischer Ethanolpräferenz zu analysieren, wurde die Funktion des Serotonin-Transporters (SERT) in olfaktorischer Ethanolpräferenz untersucht. SERT ist der Schlüsselregulator in der Serotonin-Signalweiterleitung. Er entfernt Serotonin aus dem synaptischen Spalt durch Serotonin-Wiederaufnahme in die Präsynapse. Dadurch wird die Wirkung von Serotonin im synaptischen Spalt beendet. Obwohl verschiedene *dSERT* Mutanten unterschiedlich veränderte *dSERT* Transkriptlevel aufweisen, haben Western Blots gezeigt, dass dSERT auf Protein-Ebene in allen *dSERT* Mutanten stark reduziert ist. Der Verlust der dSERT Expression korreliert mit einer veränderten Fortbewegung, da *dSERT16* Mutanten keine negative Geotaxis zeigen und das Kletterverhalten bei *dSERT18* Mutanten ebenfalls beeinträchtigt ist. Auch die olfaktorische Ethanolpräferenz der *dSERT* Mutanten wurde getestet. Die *dSERT16* Mutanten konnten sich weder für Futter mit Ethanol noch für Futter ohne Ethanol entscheiden. Dieses Ergebnis suggeriert, dass Serotonin ein negativer Regulator ist, weil erhöhte Serotoninlevel zu reduzierter Kletterfähigkeit und einem Verlust an olfaktorischer Ethanolpräferenz führt. Um die genaue Rolle von Serotonin in olfaktorischer Ethanolpräferenz zu bestimmen, wurde eine dominant-negative Version des Serotonin-transporters, welcher Serotonin nicht binden kann, in unterschiedlichen Neuronen im Fliegengehirn exprimiert, wodurch dort die Serotonin Signalweiterleitung erhöht wurde. Die Expression dieses modifizierten Transporters in Neuronen, die durch die *TPH*-GAL4 angesprochen werden, führte zu einer Verminderung der Ethanolpräferenz. Dies passiert

aufgrund der verlängerten 5-HT-Signalweiterleitung, da ein ähnlicher Phänotyp beobachtet werden kann, wenn Fliegen mit dem Serotonin-Vorläufer 5-HTP gefüttert werden, was zu einem erhöhten Niveau von 5-HT führt (Schläger, 2013). Der Präferenzphänotyp liegt nicht an Fortbewegungsproblemen, da TPH-GAL4/UAS-SERTDN Fliegen normales Geotaxis-Klettern aufweisen. Diese Ergebnisse zeigen, dass die Erhöhung des Serotoninspiegels Ethanolpräferenz unterdrückt und die Teilmenge von serotonergen Neuronen, die durch die TPH-GAL4 angesprochen werden, für olfaktorisches Ethanol-induziertes Verhalten erforderlich ist. Wenn die dSERT Funktion in SERT3-GAL4 abhängigen serotonergen Neuronen gestört wird, ist eine verminderte Ethanolpräferenz zu beobachten. Allerdings zeigen diese Fliegen robuste Fähigkeit im Klettern. Eine Untergruppe von sechs serotonergen Neuronen im IP, LP1 und SE1 Cluster wurden gefunden. Vier gemeinsame serotonergen Neuronen im IP und LP1 Cluster können nach Vergleich neuronale Expressionsmuster von SERT3-GAL4 mit TPH-GAL4 bestimmt werden. Das bedeutet, dass Ethanolpräferenz in diesen vier serotonergen Neuronen des IP und LP1 Clusters im Gehirn vermittelt wird. Überraschenderweise verändert die Expression von UAS-SERTDN in TRH-GAL4-abhängigen Neuronen, welche 83% der serotonergen Neuronen im ZNS abdecken, nicht die Ethanolpräferenz. Neben den Neuronen, die sowohl von der TPH-GAL4 als auch in SERT3 GAL4-Treiberline angesprochen werden, konnten zusätzliche serotonergen Neuronen im CSD, DP und im Abdominalganglion nachgewiesen werden. Diese Daten legen nahe, dass ein weiterer serotonerger neuronaler Schaltkreis besteht, der Ethanolpräferenz moduliert. Um zu überprüfen, dass die Präferenz nicht auf unterschiedlicher Expressionsstärke der verschiedenen GAL4 Expressionen basierte, wurde UAS-SERTDN simultan in SERT3-GAL4 und TRH-GAL4 assoziierten Neuronen exprimiert. Hierbei konnte keine Veränderung der Präferenz beobachtet werden. Ebenfalls keine Veränderung der Präferenz wurde gezeigt, wenn UAS-SERTDN in *SERT3*-GAL4/RN2-E-GAL4 Neuronen exprimiert wird. Diese Fliegen zeigten jedoch Kletterdefekte in negativer Geotaxis. Die RN2-E-GAL4 Linie treibt CSD Neurone im Gehirn und in einem Cluster im Abdominalganglion. Serotonerge Zellen im CSD Cluster und im Abdominalganglion sind daran beteiligt Ethanolpräferenz zu vermitteln.

Abschließend ist zu sagen, dass dSERT an der Modulation von olfaktorischem Präferenzverhalten und negativer Geotaxis beteiligt ist. Serotonin wirkt als negativer Modulator in Ethanolpräferenz. Erhöhter Serotoninspiegel führt zu reduzierter Ethanolpräferenz wobei vier serotonerge Neurone im IP und LP1 Cluster dieses Verhalten vermitteln. Der Präferenzphenotyp ist nicht hervorgerufen durch Defekte in der Bewegungsfähigkeit. Ein weiterer serotonerger Schaltkreis ist wahrscheinlich für die Vermittlung von olfaktorischer Ethanolpräferenz in *Drosophila melanogaster* beteiligt.

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1. Introduction

1.1 Ethanol induced behavior in *Drosophila melanogaster*

In nature, ethanol is not only present in leaves and fruits fermented by microorganisms, but also detectable in the tissue of other organism as a metabolic by-product (Holmes, 1994). The concentration of ethanol in the wild is relatively low and almost all animals can metabolize ethanol. In ethanol containing environment, such as the winery, fruit flies are frequently found. Genetic analysis showed that up to 75% disease associated genes of human have ortholog in *Drosophila* (Chien et al., 2002). Easy to husbandry and rich in genetic tools make *Drosophila melanogaster* an ideal model organism to study ethanol induced behaviors. Ethanol metabolize some time is used as a source of calories and also essential for flies to prevent ethanol toxicity (Decineni and Heberlein, 2013). However, McClure et al. (2011) reported that if flies continuously kept in more than 5% ethanol containing food, they will show decreased survival rate, smaller adult body size and delayed development.

Drosophila responses to ethanol exposure could lead to hyper activity in low concentration and sedation at higher concentration which are similar to humans and other mammals (Pohorecky, 1977). Wolf et al. (2002) developed a video based system to track fly's ethanol induced locomotion activity and found that ethanol extend the duration of ethanol induced hyperactivity. Repeated exposure to ethanol vapor to flies after their recovery can cause decrease in ethanol sensitivity which is defined as ethanol tolerance (Scholz et al., 2000). In addition to the compulsive ethanol educed response, when food choices are offered with or without ethanol flies show preference to ethanol containing food. Recent research addressed that preference to ethanol containing media is gainful for *Drosophila* to fight against its parasite wasps (Milan et al., 2012). The test of proboscis extension duration showed that naive flies get preference to ethanol contained media; this preference could be enhanced by pre-exposure to ethanol (Cadieu et al., 1999). However, the measurement for duration of proboscis extension cannot represent the real ethanol intake. Capillary feeder (CAFE) assay can quantify the real-time consumption of liquid food for single or grouped flies, which makes the CAFE assay available to test the ethanol preference precisely (Ja et al., 2007). Flies prefer food containing 5%-15% of ethanol when provide flies ethanol containing food or regular food in CAFE assay (Devineni and Heberlein, 2009). In two odor choice assay wild type flies show preference to 5% of ethanol with juice (Ogueta et al., 2010). Latter research

on ethanol associated odor preference suggested that ethanol played a rewarding role in decision making (Kaun et al., 2011).

1.2 Serotonin expression in Drosophila CNS

Initially Serotonin positive neurons in adult *Drosophila* brain are divided into 8 clusters according to their location. There are in total around 31 serotonergic neurons per hemisphere in the adult brain (Vallés and White, 1988). Later study showed that in adult fly's brain 38-41 serotonergic neurons per hemisphere were identified (Sitaraman et al., 2008). But, Alekseyenko et al. (2010) found 35 serotonergic cells pre hemisphere. Even though they used a different nomenclature than Vallés and White (1988), two new clusters amp and alp were added to total serotonergic cells. However further analysis changed the number of clusters and total cell numbers. Recently 12 different serotonergic clusters were described in adult *Drosphila* brain with total number of 40 neurons per hemisphere (Giang et al., 2011). Serotonergic clusters are summarized in Figure 1.2. The adult thoracic ganglia had been divided into pro-, meso-, meta- thoracic segment and the abdominal ganglia (AB). There were 22 serotonergic cells in larvae VNC (Huser et al., 2012). Although it cannot distinguish how many 5-HT positive cells in adult AB, it believed that the same numbers of cells in adult thoracic ganglia as in larval abdominal ganglia (AB) (Vallés and White 1988).

A

B

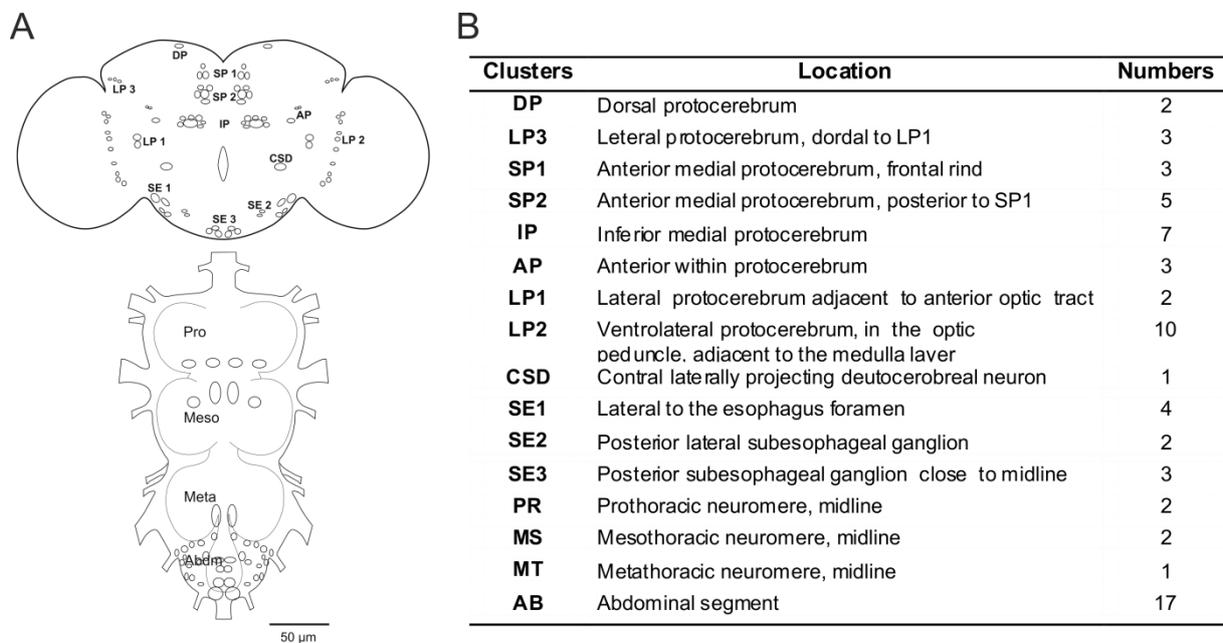| Clusters	Location	Numbers
DP	Dorsal protocerebrum	2
LP3	Leteral protocerebrum, dordal to LP1	3
SP1	Anterior medial protocerebrum, frontal rind	3
SP2	Anterior medial protocerebrum, posterior to SP1	5
IP	Inferior medial protocerebrum	7
AP	Anterior within protocerebrum	3
LP1	Lateral protocerebrum adjacent to anterior optic tract	2
LP2	Ventrolateral protocerebrum, in the optic peduncle. adiacent to the medulla laver	10
CSD	Contral laterally projecting deutocerobreal neuron	1
SE1	Lateral to the esophagus foramen	4
SE2	Posterior lateral subesophageal ganglion	2
SE3	Posterior subesophageal ganglion close to midline	3
PR	Prothoracic neuromere, midline	2
MS	Mesothoracic neuromere, midline	2
MT	Metathoracic neuromere, midline	1
AB	Abdominal segment	17

Figure 1.2 Serotonergic cell cluster in the adult CNS of *Drosophila*. (A) Cell numbers in each cluster is the average of 5-HT positive cells from different GAL4. (B) Abbreviation, location and cell number of clusters. Drawing was modified after (Vallés and White, 1988; Giang et al., 2011).

1.3 Serotonin involved behaviours

Biogenic amine serotonin (5-HT, 5-Hydroxytryptamine) is not only a neurotransmitter, but also acts as neuromodulator in the brain (Bunin et al., 1999). Serotonin it is associated with many different behaviors. It had been shown that serotonin is involved in aggression of both vertebrate and invertebrate (Popova, 2006; Kravitz and Huber, 2003). Dierick and Greenspan (2007) found that increased serotonin level in fly's brain via feeding 5-HT precursor 5-HTP enhances its aggression. Selectively activate serotonergic neurons by expressing dTRPA1 in *TRH*-GAL4 lines will also provoke the increase of fly's aggression (Alekseyenko et al., 2010). Therefore, elevation of 5-HT level causes increase of aggression. The heat-box treatment of *Drosophila* demonstrated serotonin is necessary for place memory (Sitaraman et al., 2008). The relation between serotonin and sleep was also been clarified as excess serotonin decrease light response in *Drosophila* (Yuan et al., 2005). Neuronal 5-HT level is also important for modulating feeding behavior because increased serotonin depresses feeding behavior (Neckameyer, 2010). Besides influencing physical behaviors, one important role of serotonin is that it can modulate fly's olfactory learning and memory. Pharmacologically block serotonin receptors reduces olfactory memory performance in *Drosophila* suggested that serotonin is involved in olfactory memory (Johnson et al., 2011). Sitaraman et al. (2008) showed that decreased 5-HT level in *Drosophila* CNS reduces memory performance. Inhibit 5-HT synthesis or release from DPM neurons disturb fly's intermediate-term memory (Lee et al., 2011).

1.3.1 Serotonin and locomotion

The central complex is the high brain center for controlling locomotor behaviors which includes walking speed in negative geotaxis in *Drosophila* (Strauss and Heisenberg, 1993). The structure is heavily innervated by serotonergic neurons (Ginag et al., 2011) suggesting that 5-HT plays an important role in the regulation of locomotion. In the *Drosophila* larvae, serotonin modulates the locomotor output in response to light (Rodriguez and Campos, 2009). dVMAT larval mutants also show decreased locomotion (Simon et al., 2009). Serotonin level might be important for locomotor behaviors since over expression of dVMAT in both serotonergic and dopaminergic neurons enhance locomotion in adult fruit fly (Chang et al., 2006). Lack of neuronal serotonin can cause a reduction of female activity (Neckameyer et al., 2007). Whereas flies treated with cocaine-an inhibitor of SERT resulting in increase of

5HT level in synaptic cleft and showed increased motor activity after cocaine treatment (Chang et al., 2006). Contradict results were generated from different labs about the relationship between serotonin level and locomotion. For example, increased serotonin level by fed *Drosophila* 5-HTP caused reduced locomotion activity (Yuan et al., 2006). Even in different regulation levels of serotonin signaling, data of locomotor activity are not consistent. Feeding *Drosophila* reserpine which inhibits dVMAT transport activity decreases locomotion (Chang et al., 2006). Serotonin receptors play a role in locomotion as well, since pharmacologically block of d5-HT7 caused an increased locomotion (Becnel, et al., 2011). Simon et al. (2008) observed homozygous dVMAT flies have decreased response to negative geotaxis climbing. There is a high chance that serotonin plays an important part in negative geotaxis.

1.3.2 Serotonin is implicated in the processing of olfactory information

Ethanol is an odor that is elicited from fermenting fruits. Olfactory ethanol preference depends on an olfactory stimulus (Ogueta et al., 2010). Odor is received at the level of olfactory receptor neurons (ORNs) localized at the antenna and maxillary pulp of the fly. ORNs are bipolar neurons that have dendritic projection on the sensilla which localized on the third antennal segment and axonal projection extending into the brain (Ache and Young, 2005). In insects, ORNs will form the first synaptic connection within the antennal lobe which is the analogy of vertebrate's olfactory bulb. In *Drosophila*, each olfactory receptor neuron only expresses one odor receptor (OR) (Couto et al., 2005). The total *Drosophila* odorant receptors are encoded by 57 genes and one ORN only expresses one receptor gene (Vosshall et al., 2000). According to odor response, there are up to 50 different ORN types which most of them can response to multiple ligands (Wilson, 2013). Depend on different odorants or ORs, after odor molecules interact with ORs on membranes of ORNs dendrites the ORNs could have either excitatory or inhibitory responds (Hallem et al., 2004). ORNs that express the same odorant receptor converge into neuropil and then synaptically connect with both local interneurons (LNs) and projection neurons (PNs) in the same glomeruli. The projection neurons send the information from glomerulus into a higher brain centers such as mushroom body and lateral horn (Keene and Waddell, 2007). Local interneurons mainly exert excitation or inhibition role of PNs response (Silbering and Galizia, 2007; Silbering et al., 2008; Gaudry et al., 2012).

Serotonergic innervations are found at the olfactory pathway antennal lobes and mushroom

body. One single serotonergic neuron CSD which send branches to antennal lobe and higher brain center had been described (Roy et al., 2007). In moth *Manduca sexta*, CSD neuron also has similar projection pattern like in *Drosophila* (Dacks et al. 2006). Two DPM neurons innervating to mushroom body were also serotonergic neurons (Lee et al., 2011). Serotonin receptor 5HT-1A and d5HT-1B is expressed in *Drosophila* mushroom body (Yuan et al., 2006; Yuan et al., 2005).

In other insects, evidence showed the involvement of 5HT in olfactory information processing at the level of antennal lobes. In moth, low levels of serotonin reduce the antennal lobe excitatory response to antenna electronic stimulation, however high concentrations increase the responses (Kloppenburg et al., 1995). In addition, serotonin increased neuronal responses in projection neurons to pheromone stimulation (Kloppenburg et al., 1999). In silk moth, serotonin can enhance glomerulus responses to antennal nerve stimulation (Hill et al., 2003) and serotonergic neurons are directly innervated into ALs in other insects (Dacks et al., 2006; Roy et al., 2007), so serotonin might modulate projection neurons and local interneurons at the same time. A similar serotonin immunoreactive neuron branching to lateral accessory lobe (LAL), central body and calyces of the mushroom body was found in silk moth; the soma is at the posterior portion of the lateral cell cluster of AL and response to mechanosensory stimuli (Hill et al., 2002). Serotonin was proved to increase the AL response to odor by increasing subset of AL unite firing rate and duration (Dack et al., 2008). In *Drosophila*, serotonin can enhance certain odorant caused responses of projection neurons in antennal lobe, as well as local interneurons (Dacks et al., 2009).

Serotonin acts as a neuromodulator in olfactory induced behaviors in *Drosophila*. Lee et al. (2011) showed that serotonin is required for aversive olfactory induced memory and therefore DPM neurons innervating the mushroom body are specifically needed. Serotonin could modulate olfactory learning by increasing or decreasing serotonin level. Dopa decarboxylase (*Ddc*) is an important enzyme for serotonin synthesis. *Ddc* mutant flies exhibited diminished olfactory learning (Tempel et al., 1984) which is due to the lack of serotonin synthesis. Serotonin also plays a role in olfactory aversive learning and memory, since pharmaceutically block of *Drosophila* serotonin receptors 5-HT$_1$, 5-HT$_2$ and 5-HT$_7$ disturbe aversion memory formation (Johnson et al., 2011). Serotonin is also required for appetitive olfactory memory, since block of serotonin release in serotonergic neurons dramatically reduces fruit fly's appetitive olfactory memory performance (Sitaraman et al., 2012).

1.4 The Serotonin transporter

1.4.1 The structure of serotonin transporter

To better understand the structure and function of the serotonin transporter, *Drosophila melanogaster* serotonin transporter (dSERT) was cloned. The *dSERT* gene is located on the second chromosome. The 3.1 kb transcript is translated into 622 amino acid resulting in a protein with a predicted molecular mass of 69kDa (Corey et al., Demchyshyn et al., 1994). Further hydropathic analyses suggest that dSERT contains 12 putative trans-membrane domains (TMD) and both N and C termini are in cytoplasm side (Fig.1.4). The TMD3 and TMD4 are connected via large hydrophilic loops (Blakely et al., 1994). The human serotonin transporter (hSERT) and rat serotonin transporter (rSERT) share 92% identity of the SERT structure (Ramamoorthy et al., 1993; Blakely et al., 1991). The dSERT also displays high homology to rat (52%) and human (53%) serotonin transporter (Corey et al., 1994).
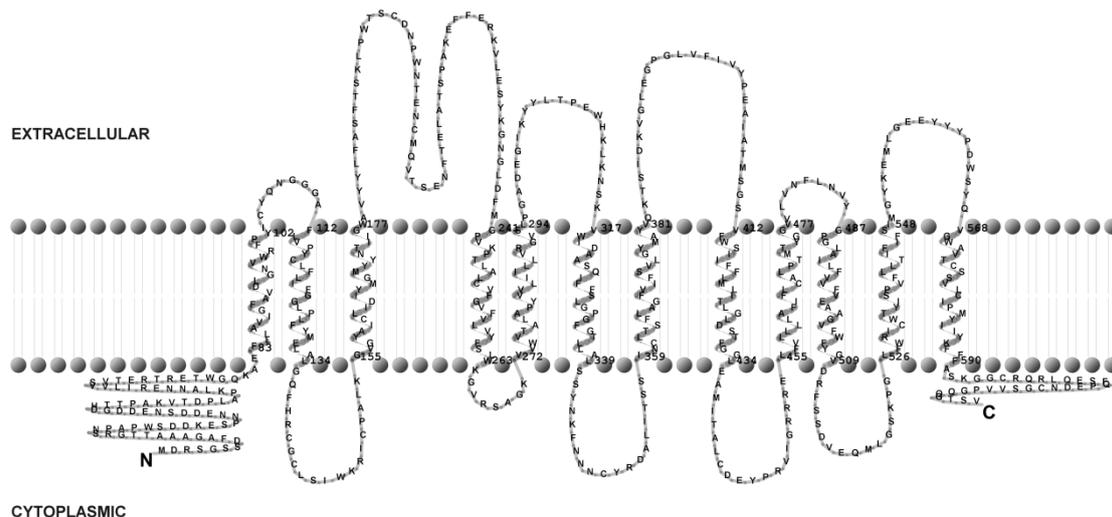
Figure 1.4 The structure of dSERT. The 622 amino acids of dSERT form 12 predicted transmembrane domains. The C termini and N termini are localized in the cytoplasmic region (Modified from Jhamna Magsig).

1.4.2 Serotonin transporter expression

The SERT is localized in the presynaptic membrane and terminates 5-HT transmission via transporting it back to the synapse. In addition SERT was also detected in axons of rat's brain (Zhou et al., 1998). In *Drosophila* the first *dSERT* mRNA can be detected at stage 15 of embryonic development which is earlier than 5-HT receptor appearance (Demchyshyn et al., 1994). The *dSERT* expression was found in the subesophageal, thoracic and abdominal

ganglion as well as in the brain region (Demchyshyn et al., 1994). In rodents and human, SERTs are not only found in the central nerve system (CNS) but also in platelet and pulmonary membranes (Qian et al. 1995). RNA hybridization experiments showed that different mRNAs are expressed in different tissues; however both neuronal and non-neuronal hSERTs are encoded by the same gene (Ramamoorthy et al., 1993). In *Drosophila* dSERT anti-sense riboprobe labeling cells are consistent with serotonergic clusters SE2, SP1 and LP1 cells in adult brain (Thimgan et al., 2006). This result suggested that SERT and 5-HT are expressed in same set of cells. Recently it was shown that in the larval and adult CNS dSERT is exclusively expressed in serotonergic cells (Giang et al., 2011).

1.4.3 The function of serotonin transporter

The dSERT is a specific 5-HT transporter, since other substrates such as tyramine, octopamine, histamine, dopamine and norepinephrine did not compete with 5-HT from uptake by dSERT (Demchyshyn et al., 1994). In addition the dSERT showed decreased affinity to antidepressant, such as fluoxetine and clomipramine in comparison to the mammalian serotonin transporter (Demchyshyn et al., 1994; Corey et al., 1994). In contrast, dSERT is more sensitive to cocaine than the mammalian serotonin transporter (Corey et al., 1994). The serotonin transporter is embedded into the membrane of pre-synapses and removes serotonin from synaptic cleft. Therefore SERT determines the duration of serotonin effect on post-synapses serotonin receptors. However, the mechanism of how serotonin transporter reuptakes serotonin from synaptic cleft has not been truly understood. At least two models exist explaining the action of the 5-HT transport by SERT.

One theory is summarized into an alternate access model. Both symport and antiport of loaded molecules are involved in 5-HT transport in this model (Rudnich, 2006). Na^+ and Cl^- are required to reuptake 5-HT from the synaptic cleft by the SERT (Hoffman et al., 1991). Similar to the human serotonin transporter, the dSERT depends also on Na^+ for 5-HT uptake (Ramamoorthy et al., 1993; Corey et al. 1994). There were debates about whether K^+ also coupled with 5-HT transportation. When internal K^+ concentration is higher than external, a 5-fold of 5-HT accumulation than steady state could be detected (Nelson and Rudnick, 1979). Even when K^+ is absent internal H^+ ions can boost 5-HT influx (Keyes and Rudnick, 1982). In summary Na^+ and Cl^- is transporter into the cell whereas K^+ or H^+ are transported to the exterior to drive 5-HT transport (Rudnick and Clark, 1993). Furthermore dSERT might act as

a serotonin channel. Corey et al. (1994) firstly detected inward currents when using dSERT expressing oocytes to absorb 5-HT. Is this a characteristic of a channel? They also found that current increased 2.4-fold between -40 and -80 mV. Therefore it is thought that serotonin uptake is voltage-independent. External 5-HT application could lead to inward current indicating the serotonin transporter does not depend on membrane potential to function (Mager et al., 1994). This is consistent with the idea the SERT could act as a channel. Similarly, the application of 5-HT to dSERT cRNA-injected oocytes leads to an inward current (Galli et al., 1997). This current was reduced by paroxetine- a serotonin transporter inhibitor. At the same time, small leakage current was record in the absence of 5-HT. However, voltage dependent dSERT uptake is independent of dSERT expression or 5-HT level (Galli et al., 1997) also showed that 5-HT induced transport and channel opening are correlated. Petersen and DeFelice (1999) propose dSERT function as serotonin permeable channels, since dSERT can increase 5-HT level continuously up to 0.3mM when exposed to high 5-HT concentration.

1.4.4 Drosophila serotonin transporter modulation

The mammals and *Drosophila melanogaster* SERT share high structural and functional homologies (Blakely et al., 1991; Ramamoorthy et al., 1993; Demchyshyn et al., 1994; Zahniser and Doolen, 2001). For example, ectopically expression of UAS-dSERT in TH dependent neurons, dSERT uptaken extracellular 5-HT was observed (Park et al., 2006). This finding is consistent with reduced 5-HT expression in the larval brain after cocaine administration (Borue et al., 2010). Inhibition of SERT function by cocaine can prolong 5-HT signaling (Borue et al., 2009). That is an indication of serotonin pool in *Drosophila* is not only determined by 5-HT synthesis but also reuptake. It is also though that dSERT reuptake is important for rapid replenishment of 5-HT releasable pool (Borue et al., 2010). The serotonin transporter modulates the quantity and duration of 5-HT and serotonin receptor interaction. At the same time, the function of SERTs is regulated by other factors than 5HT. The activation of protein kinase C (PKC) caused a reduction of 5-HT uptake in HEK293 cells, this effect is due to the internalization of cell surface hSERT protein (Qian et al., 1997). The same phenomenon was also found in platelet. Furthermore, after longer time (30-min) activation of PKC leads to a decreased cell surfaced SERT and increase of intracellular SERT (Jayanthi et al., 2005). There are certain factors that have potential to influence SERT location. Syn1A which is short for plasma membrane SNARE protein syntaxin 1A is associated with SERT and alters the

sub-cellular location of SERT (Haase et al., 2001). During interaction of Syn1A binding at the N-terminal tail of rSERT in oocyte cells, SERT conducting states can be changed (Quick, 2003). SERT activity can also be boosted via activation of p38 MAPK without change of cell surface density (Zhu et al., 2005). In addition to interaction at the N terminus of SERT, the carboxy terminal also interacts with other factors. For example, SERT decreased cell surface localization and 5-HT uptake when co-expressing it with neuronal nitric oxide synthase (nNOS) (Chanrion et al. 2007).

1.5 Serotonin signaling modulation

In serotonin signaling, serotonin transporter (SERT) can terminate serotonin transmission in synaptic cleft through reuptake serotonin to cytoplasm. Thus, serotonin reuptake plays an important role in regulating 5-HT transmission. Some other factors, such as tryptophan hydroxylase (TPH), monoamine oxidase (MAO), serotonin receptors and *Drosophila* vesicular monoamine transporters (dVMAT) which can modulate serotonin level are also crucial for neuronal serotonin signaling control. These factors work on different aspects to regulate quantity, location and duration of serotonin transmission.

1.5.1 Tryptophan hydroxylase determines serotonin synthesis

Biogenic amine serotonin is synthesized in two steps. Firstly tryptophan hydroxylase (TPH) converts tryptophan to 5-hydroxytryptopan which is the rate limiting step of serotonin synthesis. Then 5-hydroxytryptopan is converted into serotonin by dopa decarboxylase (DDC).

In mammalian there are two isoforms of TPH which are encoded by the genes *Tph1* and *Tph2*. TPH1 is expressed in the periphery and TPH2 is exclusively expressed in CNS (Zhang et al., 2004; Walther et al., 2003). In *Drosophila,* there are also two different tryptophan hydroxylase enzymes for serotonin synthesis which encoded by two different genes; they have been named DTPH and DTRH according to their primary roles and expression (Coleman and Neckameyer, 2005). DTPH was termed as DTRHn because of its neuronal expression and function and it is also the homology to mammalian TPH. In early embryonic stage DTPHu expression could be detected, but DTRHn appears until late embryogenesis (Neckameyer et al., 2007). Immunostaining studies revealed that DTPHn is exclusively expressed in

serotonergic neurons in the larval CNS (Neckameyer et al., 2007). In the adult brain TRH-immunoreactive (TRH-IR) cells are located in the same position as serotonergic cells (Bao et al., 2010). Newly synthesized 5-HT by TPH is important for proper serotonin signaling. Inhibiting DTRH hydroxylase activity by *p*-chlorophenylalanine (PCPA) can lead to serotonin content decreased in *Drosophila* CNS (Borue et al., 2010).

Experiments of TPH mutants also confirmed the idea that TPH is required for serotonin level in the cells. In DTRHn null mutants, 5-HT immunoreactivity level is reduced in larval CNS and mutants show defects in feeding and locomotion behaviors (Neckameyer et al., 2007). Mammalian TPH malfunction can cause abnormal behaviors as well. *Tph1* mutant mice display cardiac dysfunctions (Côté et al., 2003). Analyses of loss of function of human hTPH2 show correlation with defect of serotonin synthesis in brain and unipolar major depressions (Zhang et al., 2005).

1.5.2 VMAT is crucial for serotonin release

After serotonin synthesis, 5-HT is transported via the vesicle monoamine transporter (VMAT) into secretory vesicles. VMAT works as a neurotransmitter transporter; it can pack neurotransmitters into secretory vesicles for regulating exocytotic secretion (Liu and Edwards, 1997). After neuronal activation the vesicles merge with the pre-synaptic membrane and monoamines including the serotonin are released into the synaptic cleft. In mammals two different VMATs have been firstly identified which named as VMAT1 and VMAT2 (Peter et al., 1992). Both VMATs recognize monoamines as substrates, even though VMAT1 has less affinity than VMAT2 (Peter et al., 1994). In *Drosophila* two isoforms DVMAT-A and B which derived from a single gene were reported (Greer et al., 2005). Since DVMAT-A internalization rate of neurotransmitter is much higher than DVMAT-B, it has been suggested that DVMAT-A is likely to transport dopamine, serotonin and octopamine into vesicle (Greer et al., 2005). In the mice VMAT2 is expressed in dopamine, norepinephrine, and serotonin neurons of the CNS (Peter et al., 1995).

Colocalization studies of DVMAT-A with TH and 5-HT in larval CNS also revealed that DVMAT-A is expressed in serotonergic SP1, SP2 and IP clusters and dopaminergic DL1, DL2 clusters which supports the idea that DVMAT transports DA and 5HT (Greer et al., 2005). DVMAT-A and serotonin colocalize in 12-14 cells in LP2 cluster of adult fly's brain and over expression of DVMAT-A in serotonergic and dopaminergic neurons leads to an increased

locomotion activity (Chang et al., 2006). DVMAT mutant flies can survive better under a low population density. In addition, DVMAT mutants show reduced fertility and impaired geotaxis behavior (Simon et al., 2009). This data supported by recent pharmacological study which inhibiting dopamine transporter (DAT) with reserpine resulting in a decrease of locomotion and fertility in *Drosophila* (Chang et al., 2006). These results add solid evidence that DVMAT involved in modulating monoamine release and storage induced behaviors.

1.6 Aims

Odor invoked decision making is vital for insects to find food and mating patter. Wild type flies showed preference to 5% of ethanol containing food (Ogueta et al., 2010). However, the mechanism of ethanol induced preference is not clear. To investigate whether serotonin plays a role in ethanol induced preference, the key regulator of serotonin signaling − the serotonin transporter (*dSERT*) was mutated by generating *dSERT* mutant (Kaiser, 2009). RNA expression pattern showed that *dSERT10* and *dSERT16* have nearly no *dSERT* transcript, but in *dSERT18 dSERT* expression was up regulated (Ruppert, 2013). This result left one question- what is the dSERT protein level in these mutants? To analyze the consequences of altered transcript level on protein expression, western blot analysis were done. After confirmation of the dSERT protein expression, behavior test for ethanol preference were performed to understand the relation between dSERT level and ethanol preference. Beside dSERT mutants another tool UAS-*SERTDN*-GFP which could specifically disturb *dSERT* function in GAL4 dependent neurons by expression of a dominant negative version of dSERT was also available (Ritze, 2007). With the help of UAS-*SERTDN*-GFP it is possible to identify which set of neurons are responsible for ethanol educed preference. Therefore, different serotonergic GAL4 driver lines were crossed with this construct and then tested in two choice assays. To visualize the neurons that can drive the expression of UAS-*SERTDN*-GFP, specific GAL4 driver lines were crossed with UAS-mCD8-GFP and the colacalization of GFP and 5-HT in adult CNS was analyzed. Combining the behavioral result and neuroanatomy localization will provide a better clue to understand the mechanism of ethanol induced decision. If serotonin can be proofed to be involved in ethanol induced preference, further studies on the function of serotonin in pre-synapse should also be performed. One way to test serotonin function in pre-synapse is to alter serotonin level by expressing genetic tools such as UAS-SERT-GFP and UAS-dVAMT in different serotonergic neurons. Another way is to artificially activate serotonergic neurons by depolarizing ion channel using an UAS-*Chr2*

transgene.

Homozygote *dVMAT* mutant flies have impaired negative geotaxis behaviors (Simon et al., 2009). Since dVMAT is required for dopamine, serotonin and octopamine vesicular storage, there is a big chance that serotonin plays a role in negative geotaxis. *dSERT* mutants need to be tested to verify whether serotonin is required in negative geotaxis. In the same time, flies expressing UAS-*SERTDN*-GFP were crossed to different serotonergic GAL4 drive lines to test for geotaxis to know the exact neurons that might controlling the behavior.

2 Material and Methods

2.1 Material

2.1.1 Solutions and Chemicals for immunostaining

PBS:	NaCl	137 mM
	KCl	2.7 mM
	Na_2HPO4	10.0 mM
	KH_2PO4	2.0 mM
	pH 7.4	
Drosoplila Ringer:	NaCl	110.00 mM,
	KCl	4.7 mM
	$MgCl_2$	20.00 mM
	Na_2PO4	0.35 mM
	KH_2PO4	0.74 mM
	pH 7.4	
Blocking solution:	Goat Serum	5.0 %
	BSA	2.5 %
	PBS	1.0 X
Reaction buffer:	Goat Serum	0.5 %
	BSA	0.25 %
	NaCl	2.0 %
	Triton X-100	0.1%
	PBS	1 X

2.1.2 Solutions and Chemicals for western blot

Homogenizer buffer A:	NaCl	10mM
	HEPES, pH 7.5	25mM
	EDTA	2mM
	Complete mini	1X
Homogenizer buffer B:	NaCl	10mM
	HEPES, pH 7.5	25mM
	cOmplete mini	1X
CHAPS:	2% CHAPS in ddH_2O	

RIPA with inhibitors:	HEPES	20 nM
	NaCl	350mM
	Glycerol	20%
	$MgCl_2$	1mM
	EDTA	0.5mM
	EGTA	0.1mM
	NP-40	10%
	Protease inhibitors	10%
	ddH_2O	
4X SDS loading buffer:	Tris pH 6.8	250nM
	SDS	8.0%
	Glycerol	40%
	Bromophenol Blue	0.4%
Coomassie Solution:	Coomassie Brilliant blue	0.5%
	Methanol	50%
	Acetic Acid	7.0%
Destaining Solution:	Methanol	50%
	Acetic Acid	7.0%
10X Tris Glycin Buffer:	Glycine	1.92M
	Tris	0.25M
TBST:	Tris	50mM
	NaCl	150mM
	Tween 20	0.05%
	pH 7.6	
Running Buffer:	Tris Glycin Buffer	1X
	SDS	0.1%
Transfer Buffer:	Tris Glycin Buffer	1X
	Methanol	20%
Stacking Gels:	Acrylamide mix	30%
	Tris pH 6.8	1.0M
	SDS	10%
	APS	10%
	TEMED	0.1%

Resolving Gels:	Acrylamide mix	30%
	Tris pH 8.8	1.5M
	SDS	10%
	APS	10%
	TEMED	**0.2%**

2.1.3 Solutions and Chemicals for PCR

Homogenizing buffer:	EDTA	50mM
	NaCl	100mM
	SDS	0.5%
	Tris pH=8.0	100mM

2.1.4 Antibodies

Primary Antibodies

Name	Dilution	Company
Rat anti-5HT	1:100	Millipore
Rabbit anti-dSERT	1:1000	Eurogentec
Rabbit anti-TH	1:500	Millipore
Rabbit anti-5HT	1:1000	Millipore
Chiken anti-GFP	1:1000	GeneTex
Mouse anti-nc82	1:20	Invitrogen
Mouse anti-Myc	1:50	Developmental Studies Hybridoma Bank

Secondary Antibodies

Name	Dilution	Company
Goat anti-rat Cy3	1:200	Jackson Immunoresearch
Goat anti-rabbit Cy3	1:200	Jackson Immunoresearch
Goat anti-chicken AlexaFluor488	1:1000	Invitrogen
Goat anti-mouse AlexaFluor546	1:500	Invitrogen
Goat anti-rabbit AlexaFluor633	1:500	Invitrogen

2.1.5 Fly Stocks

Name	Genomic Localization	Donator
Canton-S	Wild type	Bloomington
W[1118]	1. Chromosome	Bloomington
dSERT1	2. Chromosome	Andrea Kaiser
dSERT4	2. Chromosome	Andrea Kaiser
dSERT10	2. Chromosome	Andrea Kaiser
dSERT16	2. Chromosome	Andrea Kaiser
dSERT18	2. Chromosome	Andrea Kaiser
Sp/CyO;TM2/TM6	2; 3. Chromosome	Bloomington
UAS-Brainbow;UAS-Brainbow	2;3.Chromosome	Bloomington
y,w,Cre;Sna/CyO	X.Chromosome	Bloomington
y,w,Cre;+;D[*]/TM3,sb	X.Chromosome	Bloomington
*y,w,hsflp;*UAS,*cd2y[+],mCD8*	X; 2.Chromosome	Wong et al.,2002
*norp*A[1];UAS-*ChR2*;UAS-*ChR2*	X; 2; 3. Chromosome	Bellmann et al., 2010
LexAop-GFP[11]*; UAS*-GFP[1-10]	2;3.Chromosome	Gordon and Scott, 2009
Or83b-LexA	3. Chromosome	Lai and Lee, 2006
TPH-GAL4	2. Chromosome	Park et al., 2006
TRH-GAL4	3. Chromosome	Alekseyenko et al., 2010
SERT3-GAL4	2. Chromosome	Andrea Herb , 2005
RN2-E-GAL4	3. Chromosome	Fujioka et al., 2003
RN2-P-GAL4	2. Chromosome	Fujioka et al., 2003
C316-GAL4	3.Chromosome	Waddell et al., 2000
UAS-*DVMAT*	2.Chromosome	Krantz et al., 2006
UAS-*SERT*-GFP	X. Chromosome	Hirsh et al., 2005
UAS-*mCD8*-GFP	X; 2; 3. Chromosome	Lee and Lou, 2001
RN2-P-GAL4/*CyO;Or83b-LexA/TM6*	2;3.Chromosome	Li Xu
y,w,Cre;TPH-GAL4	2. Chromsome	Li Xu
y,w,Cre;+;TRH-GAL4	3. Chromosome	Li Xu
y,w,Cre;TPH-GAL4	2. Chromsome	Li Xu
UAS-*SERT*-GFP;*dSERT10*	X; 2. Chromosome	Henrike Scholz

dSERT10;TRH-GAL4	2; 3. Chromosome	Li Xu
SERT3;TRH-GAL4	2; 3. Chromosome	Li Xu
SERT3;RN2-GAL4	2; 3. Chromosome	Li Xu

To reduce the impact of the genetic background in behavioral experiments, all fly lines were back-crossed to the W^{1118} line for five generations. In order to generate experimental flies, necessary cross were set up, then next generation male flies of appropriate age and numbers were collected.

All experiments are carried out at 25°C or room temperature, except otherwise stated. All experimental flies were raised on standard agar-cornmeal-yeast food at 25 °C and 60% relative humidity on a 12h/12h light-dark cycle.

2.2 Methods

2.2.1 Ethanol Preference

This method is used to test decision making of flies from two different odors. For each set up 80 male flies aging less than five days were collected and kept at 25 °C for 48 hours prior to use. The juice used in preference assay is organic apple mango juice which contains 25% of mango and 75% of apple (Alnatura). It will be mentioned in the text if different odors were used in different experiments.
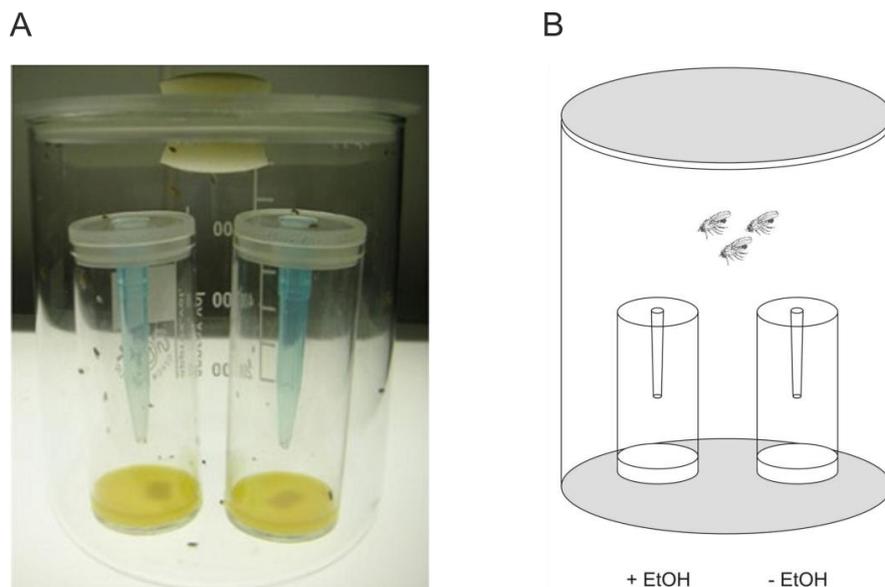

Figure 2.2.1 Ethanol preference assay (Ogueta et al., 2010)

To counteract the juice variation of different batches, each time 10 bottles of juice were fully mixed together in a big container then stored at -20°C in 50ml falcon tubes. One hour before experiment, frozen juice was thawed in cold water bath then mixed carefully.

The preference trap was modified from Larsson et al. (2004). Experimental setting of ethanol preference was according to the description of Ogueta et al. (2010) (Fig. 2.2.1). Each 1000mL beaker contains two odor traps, one of them filled with 1.5mL apple mango juice, the other one was filled with 1.5mL fresh made 5% ethanol in apple mango juice. The vial was sealed by Plexiglas cover which includes a pipette tip in its middle. For pipette tip, cut the diameter of its tip to 1.8mm to make sure flies can only go into vials but not move out. Each experimental assay was set up at 4-6 pm and kept the setting on cold light for 16 hours. Flies trapped in both juice vials and 5% ethanol vials were recorded to calculate Preference index (PI), as following equation:

$$PI = \frac{[\text{Number of flies in 5 \% ethanol juice}] - [\text{Number of flies in plain juice}]}{\text{Total number of flies}}$$

Only the groups in which more than 70 flies were trapped in two vials were evaluated. In the case where flies cannot decide, the PI is still counted but the numbers of the flies left outside of the traps will be mentioned separately.

2.2.2 Negative geotaxis assay

The method and the apparatus were modified from Kamikouchi et al. (2009). When wild type flies were given a negative geotaxis choice, majority of flies chose to climb to the upper part of the tube. Most of wild type flies finally stayed in the last two tubes (Fig. 2.2.2A). To make sure both the experimental flies and control groups get the same treatment, the set up was changed to two parallel rows of tubes (Fig 2.2.2 B). This change enabled to process two genotypes at the same time under the same condition.

For each negative geotaxis test, 40 less than 5 days old male flies were collected and kept at 25 °C for 36 hours ahead of the experiment. Flies are firstly put in tube 1 and after 5 minutes adjustment to the new environment they are knocked down to the bottom. Moved the top part of the gadget to the left immediately (1' and 1 are matched together) and kept in this position for 30 seconds. In these 30 seconds, flies will try to climb up to upper tube in response to

gravity. After this period the top part is moved to the right again and the flies were knocked down, followed by moving the top part to the left immediately again. Wait another 30sec then repeating this transfer procedure until flies have reached last tube. The number of flies in each tube is counted and used to calculate the distribution pattern. Sedate flies in refrigerator then count flies for distribution ratio. Flies in the first two tubes are count as group one, the middle two tubes as group two and last two tubes as group three. Percentage of each group is calculated as number of flies in the group divided by the total number.
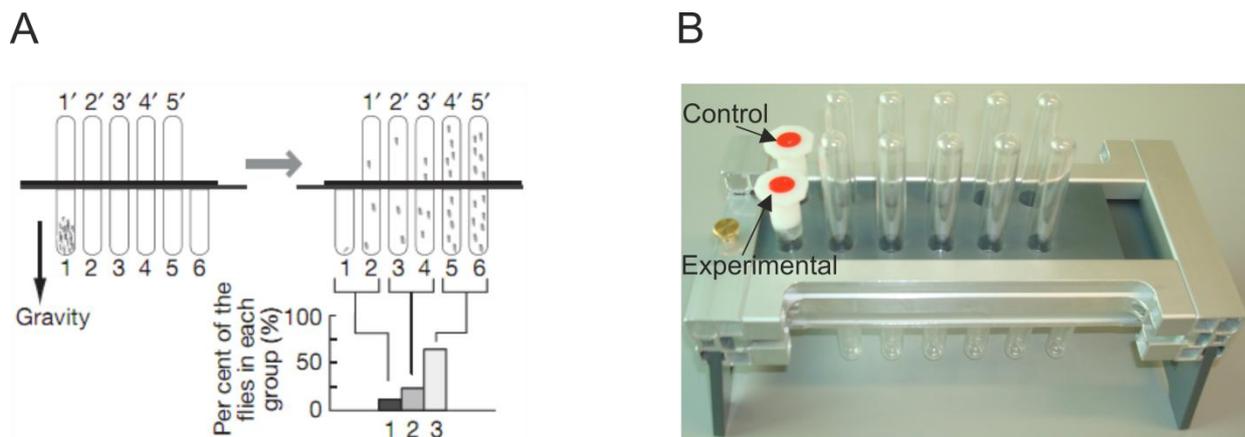
A   B

Figure 2.2.2 Climbing assay (modified after Kamikouchi et al., 2009)

2.2.3 Ethanol tolerance

Ethanol sensitivity and tolerance were measured in inebriometer (Scholz et al., 2000). The inebriometer consisted of four 122cm columns. Inside of each column is circulating ethanol vapor contains water vapor (50:45) (Fig. 2.2.3 A). Outside of columns are coated with running water to keep inside temperature at 20℃. Prior to test, two to five days old flies was collected and kept at 25℃ humidified room for 36-48 hours. Let ethanol vapor running in the columns 1.5h before test to make sure inside ethanol concentration is consistent. Population of about 100 age controlled flies was inserted into the top of column.

The sensitivity of *Drosophila* is measured by measuring their ability to maintain postural control under the ethanol vapor treatment. After being introduced into the column for certain time some flies became intoxicated then lost postural control and fell down through the oblique mesh baffles to the bottom. For each three minutes the number of flies which fell to the bottom will be recorded by light beam. Finally, total time flies spent in the column was calculated by mean elution time (MET). After first exposure to ethanol vapor, intoxicated flies

were collected. These flies recovered in 25℃ room for four hours before second ethanol exposure. Wild type flies show ethanol tolerance, since they are resistant in loss of postural control on second exposure (Fig.2.2.3 B). The tolerance is quantified as $100\times$ ([MET2-MET1]/MET1).
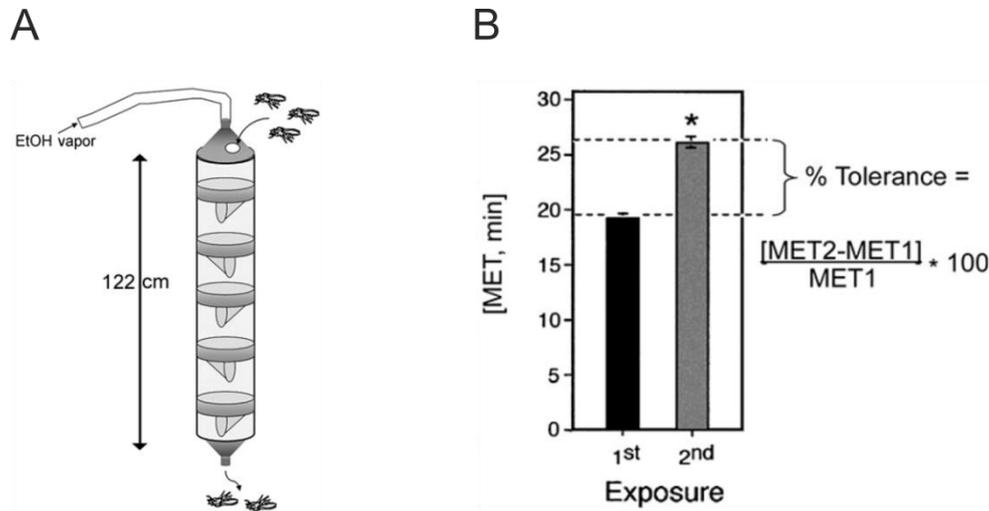

Figure 2.2.3 Ethanol sensitivity and tolerance assay (Scholz et al., 2000)

2.2.4 Light activation experiment

Experimental flies expressing $norpA^1$; UAS-*ChR2*; UAS-*ChR2* (Bellmann et al., 2010) and *SERT3*-GAL4 were bred on 150 ml of standard food containing either ethanol dissolved 150mM all-trans retinal or absolute ethanol. After hatching, 80 male flies were collected in medium food vials mixed with pure ethanol or 150mM all-trans retinal according to which food they were raised. To avoid degeneration of all-trans retinal, all food vials were surrounded with aluminum foil then kept in a dark box. Two day after rest in 25℃, 3-5 days old flies were tested for two juice odor under blue and warm white light in a dark apparatus.

Light activation set up consists of a dark chamber where flies can freely move and two odor traps filled with food odor surrounded by light isolate plastic. There is one blue diode and one yellow diode separately on top of the two odor traps that can be activated with different frequencies (Fig. 2.2.4). Flies were tested in this set up for more than16h under the following light sequence repeat of both LEDs: 40 Hz for 2s, followed by 16s with 8 Hz and 2s constant light. The intensities of the LEDs were standardized to 1800lx every time before test. For blue light illumination a LED (465-485 nm; Cree, Germany) and for yellow light illumination a

warm white LED (Cree, XLAMP, XR_E LED with 2,600 k-3,700K CCT) with a 510 nm yellow filter (HEBO, Aalen, Germany) were used. The light frequencies and sequence was controlled by program LTPFreq. After all the flies decided, numbers of flies in blue light illuminated trap and warm-white illuminated trap were counted, then the light preference was calculated as: (number of flies in blue-number of flies in warm-white)/Total numbers of decided flies.
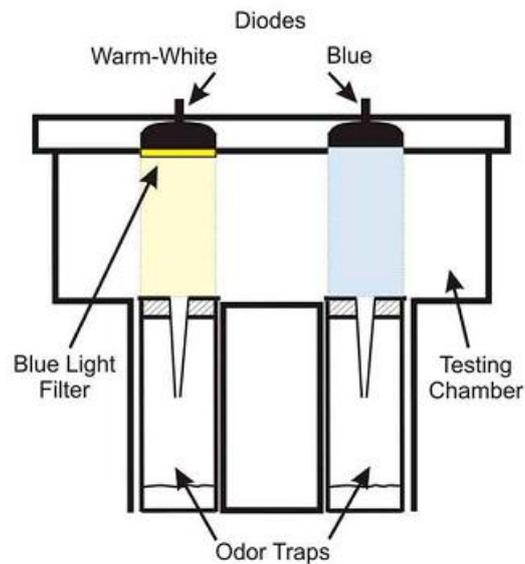

Figure 2.2.4 Light activation (Schneider et al., 2012).

2.2.5 Immunohistochemistry

The protocol for fly CNS dissection and staining is based on Wu and Luo (2006). In brief, sedated 3-5 days old male flies were kept on ice cold Petri dish. Transfer the fly in absolute ethanol for 30 sec; it was then fixed in Sylgard dish with a needle in the abdomen. The fixed fly then was covered by several drops of ice cold *Drosophila* ringer. Use forceps to remove all the legs and wings. Right after that, clean the forceps, use one to pull the proboscis and cut it off with another. Gently insert two forceps into the cavity where the proboscis used to be and hold the cuticle surrounding the cavity at opposite side. Tear the cuticle off the brain by pulling two forceps apart from each other. Afterwards, carefully remove the tissues and trachea which stick to the brain. When all the tissues were removed from the brain, slowly tear the cuticle that cover ventral nerve cords of the fly until the thoracic ganglia is seen. Clean tissues and cuticle until the whole thoracic ganglia appeared. Finally, cut all the nerves connected with CNS and put it in ice cold PBS. After dissection, CNS was fixed with

agitation in 4% formaldehyde at room temperature for 30 minutes. Samples were washed three times in 0.3% PBST (PBS with 0.3 % Triton X-100), 15 minutes each time. This was followed by keeping the CNS in blocking solution for 60 minutes at room temperature. Appropriately diluted primary antibody was applied to the tissue over night at 4°C. Prior to secondary antibody incubation over night at 4°C, samples were washed for three times with PBST for 20 minutes each time. Washing of CNS after secondary antibody incubation was done similar to washings after the primary antibody incubation. Tissue was incubated in 50 % glycerol for 30 minutes and then mounted in VectaShield (Vector laboratories) with two pieces of glass as spacer under the cover slide. In the last, nail-polish was used to seal the rim of the cover slides to keep the specimen from drying. Before scanning the specimen should be kept in a dark folder at 4°C.

2.2.6 Western blotting

Two different strategies were used to extract tissue protein. The first one described below is to extract protein from the whole cells. 20 flies' heads were collected on liquid nitrogen. Transfer frozen heads to a pre-chilled homogenizer on ice. Add 100-200μL RIPA with inhibitors to it and homogenize tissue. Incubate the homogenizer on ice for 30min then transfer homogenizer to a sterile tube. Supernatant was removed to a fresh tube after 20min of centrifuge (4°C, 15000rpm). Add SDS (with β-mercaptoethanol) loading buffer then incubate for 5min at 95°C. After cooling the sample on ice store it at -80°C before use.In the aim to get protein from both cytoplasm and membrane of the brain, samples were processed in the following order. Collect 500-1000 flies in liquid nitrogen and dissect heads by sterile mortar. Using a sterile pestle to powder the heads totally then transfer it into a homogenizer and re-suspend in buffer A. Homogenize it carefully with a glass pestle. Let the suspension solution stay in ice for 10 min, at the same time mix it occasionally. Spin down the mix at $18300x\ g$ at 4°C to get cytoplasmic protein in the supernatant. Re-suspend the pellet in buffer B thoroughly and drop 2% CHAPS in to the tube. To get more membrane protein dissolved in to the solution, keep it on ice and shake it carefully every three minutes. Collect supernatant to a new tube after spinning it down at $8000x\ g$ 4°C. Before storing the sample in -80°C with loading buffer, Bradford Assay should be done to know the protein concentration. During the SDS PAGE, 20μg of each sample was loaded in the lane. Before samples run into the resolving gel 100 Voltage is chosen then using 120V to separate the protein. Protein was transferred to Polyvinylidene difluoride membrane using a wet transfer method which was

running at 200mA for two hours in transfer buffer. Membrane was washed once after transfer then blocked with 5% milk in room temperature for one hour. Primary antibody with appropriate dilution was applied to membrane. Sample was washed with TBST for 15 minutes 3 times after overnight incubation with primary antibody at 4°C. Incubate membrane with diluted secondary antibody at room temperature for one hour then followed by 15 minutes washing with TBST for 3 times. Fresh made detection reagent (GE Healthcare) was applied to the membrane. After 5 minutes of reaction, remove the entire detection reagent applied. Autoradiography was performed in dark room using the developing machine (AGFA CURIX 60) to develop the films.

2.2.7 PCR

Genomic DNA was isolated from flies' whole body whose details will describe as below. In every eppendorf tube ten flies were collected. Tubes were kept on ice then 200μL homogenizing buffer was added to each tube and flies were homogenized gently. Samples were incubated at 70°C for 30 minutes. For each 200μL of homogenate add 28μL of 8M potassium acetate and incubate the mixture for 30min on ice. Using full speed, centrifuge the tube for 15 minutes at 4°C then transfer supernatant to new tube. Same volume of Phenol-chloroform was prepared and added to the supernatant. After mixed several times to the sample, centrifuge it for 2 minutes at room temperature. Equal volume of the top phase was removed to new tube containing chloroform. This solution was then mixed and centrifuged for 2 minutes. Top phase was transferred to a new tube and half volume of iso-propanol was added. Centrifuge the mixture at room temperature for 5 minutes and remove all the solution. Pellet was rinsed with 70% ethanol, dried and dissolved in ddH$_2$O. PCR reaction system was 50μL which contains 1μL of template, primer, dNTP, 5μL of 10 x buffers and 41μL of ddH$_2$O. The PCR program was running one cycle of initialization at 95°C for 5 minutes, 45 cycles of denaturation 95°C for 30 seconds, annealing 55°C for 30 seconds, elongation 72°C for 30 seconds and one cycle of final elongation at 72°C for 10 seconds.

2.2.8 Imaging

Before imaging, specimens were checked with Olympus BX61 fluorescence microscope and the program is Olympus Cell^F. All specimens were scanned by Zeiss 510 confocal microscope with software LSM510 META. Whole brain was scanned with 20x plan

apochromat 0.75 numerical aperture lenses. For details of different clusters 40x neofluar oil 1.3 numerical aperture lenses was used. Alexa fluor 488 was imaged with a 488nm argon laser, Alexa 446 and Cy3 was using HeNe laser, Alexa Fluor 633 was visualized with HeNe 633 laser. Detail of scanning settings: 1024 x 1024 pixels, scan speed 7, scan average number 4 and 1μm interval sequential scanning.

2.2.9 Statistical analyses and picture processing

All data were statistically analyzed using StatView 5.0.1 and Statistica 9.1. StatView nonparametric one sample sign test was used to analyze whether sample is different from zero. ANOVA Post Hoc Test was used to test the differences of different experimental groups. Student T test was used to analyze two samples in climbing assay. Zeiss LSM Image Browser Version 4.4.0.121 and Image J A.1.44 were used to analyze the localization of neuron clusters and numbers of neurons. The pictures form confocal images were stacks of Z project from Image J. Images were processed using CorelDRAW X5 and Adobe Photoshop CS5/6.

3. Results

3.1 Drosophila serotonin transporter (dSERT) is required for ethanol odour induced preference

To determine the function of 5-HT in olfactory ethanol preference, the function of the key limiting factors of 5-HT signaling the SERT should be dissected in olfactory ethanol preference. The newly generated dSERT mutants have different *dSERT* RNA expression pattern (Ruppert, 2013). However, the dSERT protein levels of these mutants still need to be investigated. Furthermore, pharmacologically fed white flies with 5-HT precursor 5-HTP led to a decrease of ethanol preference, but which neurons serotonin exert the function and what role it plays are also waiting for discovery.

3.1.1 Dramatic reduction of dSERT protein expression in *dSERT* mutants

So far only UAS-SERT was used to alter SERT gene function (Park et al., 2006) and pharmacological manipulation of SERT function. To further understand whether dSERT is involved in ethanol induced behavior and what role it plays in these behaviors, dESRT mutants were generated by P-element mutagenesis (Kaiser, 2009). The newly isolated dSERT mutants firstly need to be characterized molecular genetically to better understand the role of dSERT. Since there is no deletion detectable in revertant fly *dSERT1* (Kaiser, 2009), it is used as a genetic control. *dSERT10*, *dSERT16* and *dSERT18* mutants carry 1121bp, 1178bp and 838bp deletions respectively (Kaiser, 2009). Secondly the levels of *dSERT* transcript expression in the mutants were analyzed in comparison to w^{1118}. The *dSERT* RNA expression in *dSERT1* did not significantly different from the control (Ruppert, 2008). RNA expression of *dSERT1* showed its *dSERT* level was not altered. In *dSERT10* and *dSERT16* nearly no *dSERT* transcript was detected, but *dSERT* expression in *dSERT18* was up regulated by 190% times (Ruppert, 2013). However, the protein level of dSERT mutants is unknown. To analyze the consequences of altered transcript level on protein expression, western blot analysis using a ployclonal anti dSERT serum were performed. Western blot of Schneider cell S2 lysate was treated with three different blocking solutions to find a suitable blocking condition (Fig. 3.1.1 A). Even though expected dSERT cannot be detected in these membranes, blocking solution with 2% of NaCl gave higher resolution of the unspecific band and no difference was found between BSA with 2% NaCl and milk with 2% NaCl. So milk with 2% of milk was picked

up for blocking solution in the next blots. In order to determine the best working condition of antibody serum, firstly pre-immune serum, second bleeding and third bleeding serum was tested to analyse dSERT expression in w^{1118} flies. There was no signal in pre-immune serum membrane as well as third bleeding membrane. Some weak band showed on second bleeding membrane but the target band was hard to discern. That might be due to the antibody concentration being not right. Therefore different concentrations of second bleeding dSERT antibody were performed to figure out which one can detect the predicted dSERT band. When use second bleeding dSERT antibody with concentration of 1:20000, 1:40000 and 1:80000 none of these concentrations could make dSERT protein visible. Among these different concentrations 1:20000 membranes showed clearest unspecific band, the other two were too weak (Fig. 3.1.1 C).
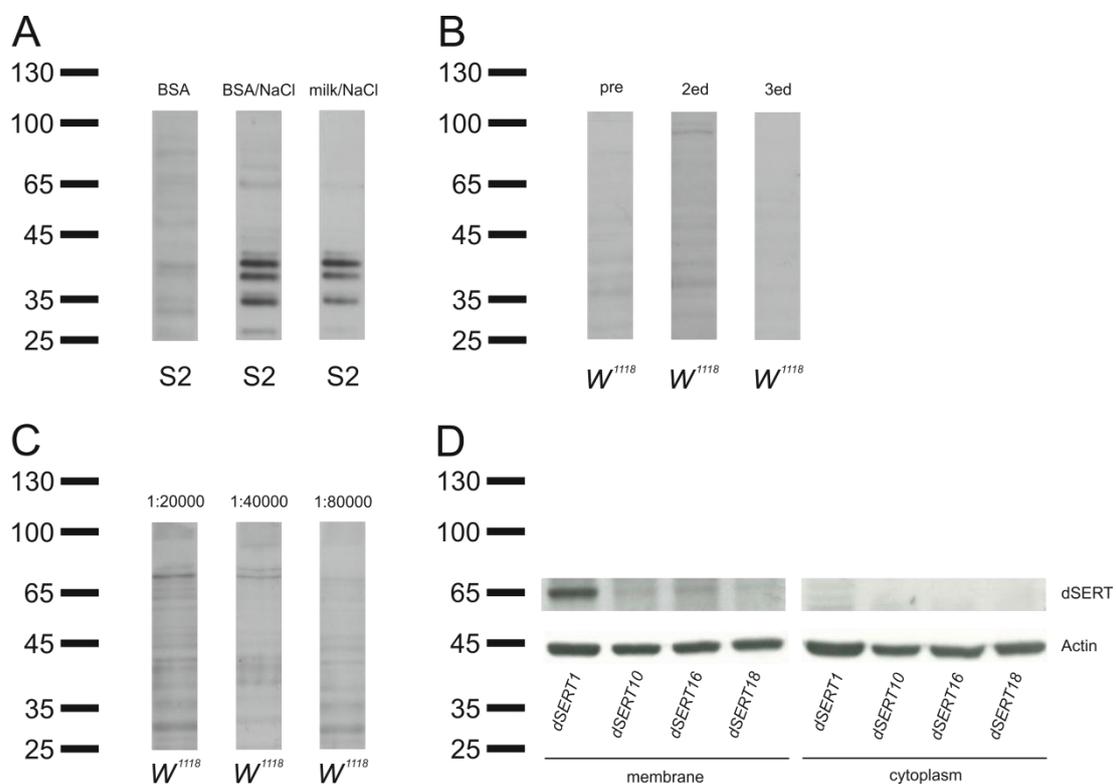

Figure 3.1.1 Serotonin transporter detection in dSERT mutants. (A) dSERT could not be detectded in whole S2 cell protein extraction. Blocking membrane with milk with 2% NaCl or BSA with 2% NaCl show more bands on the membranes. (B) Both second and third bleeding dSERT antibody was not able to recognize dSERT from whole protein extraction of 15 flies' brains. (C) Second bleeding dSERT antibody with different concentration could not visualize dSERT band from whole protein extraction of 15 flies' brains. (D) dSERT can be detected at about 65kD from membrane protein extraction of *dSERT1* flies when using 1000 heads. In dSERT mutants membrane extraction dSERT is severely reduced; no different dSERT level could be distinguished among

dSERT10, *dSERT16* and *dSERT18*. No clear band can be seen in membrane of cytoplasm protein extraction. Actin used as a loading control which shows no difference within different genotypes.

In adult fly's head, there are about 80 serotonergic neurons in total; serotonin transporter is also located in serotonergic cells (Giang et al., 2011). The protein extraction from 15 flies' heads might not provide enough protein to be detected by western blot analysis. Therefore, protein extraction of membrane and cytoplasmic protein from around 1000 flies' heads was used for western analysis. As showed in figure 3.1.1D expected protein dSERT which is about 65kD was detected from *dSERT1* membrane extraction. In all dSERT mutants, *dSERT10*, *dSERT16* and *dSERT18* nearly had no dSERT protein. dSERT level is severely reduced in dSERT mutants suggesting that *dSERT* gene deletion caused loss of SERT expression. In cytoplasmtic fraction of the protein isolation no dSERT protein could be detected, consisetnt with the idea that dSERT is mainly expressed in a membrane integrated fashion (Fig. 3.1.1 D). It can be concluded that the second bleeding dSERT antibody serum is specific and dSERT mutants are strong hypomorph.

3.1.2 dSERT mutant shows defect in ethanol odour induced behaviour

Drosophila shows preference to 5% ethanol with juice (Ogueta et al., 2010). Serotonin can enhance odorant response in the antennal lobe of *Drosophila* (Dacks et al., 2009). There is also pharmacological evidence to show that increase or decrease of serotonin level could boost or impair flies olfactory learning and memory (Lee et al., 2011). In dSERT mutants, dSERT protein on the membrane was dramatically decreased. Serotonin signalling is prolonged in these flies because of lack of reuptake. To address whether serotonin is required for ethanol induced preference, dSERT mutants were tested. There is no significant difference between dSERT1 and W1118 flies in ethanol preference ($P > 0.05$). *dSERT10* and *dSERT18* flies exhibited 42% of preference to 5% of ethanol which is similar as *dSERT1* (Fig. 3.1.2A). 48% of dSERT16 flies lost the ability to go into the trap (Fig. 3.1.2B), thus preference could not be calculated. To test the reason that made *dSERT16* could not decide, food odour versus 5% ethanol with water was offered. Wild type flies prefer food odour, *dSERT16* again had about 40% of flies could not decide (Fig. 3.1.2C). This suggests that dSERT is required for odour induced preference. Furthermore, severe lack of dSERT protein on cell membrane could destroy fly's initiation for odours.
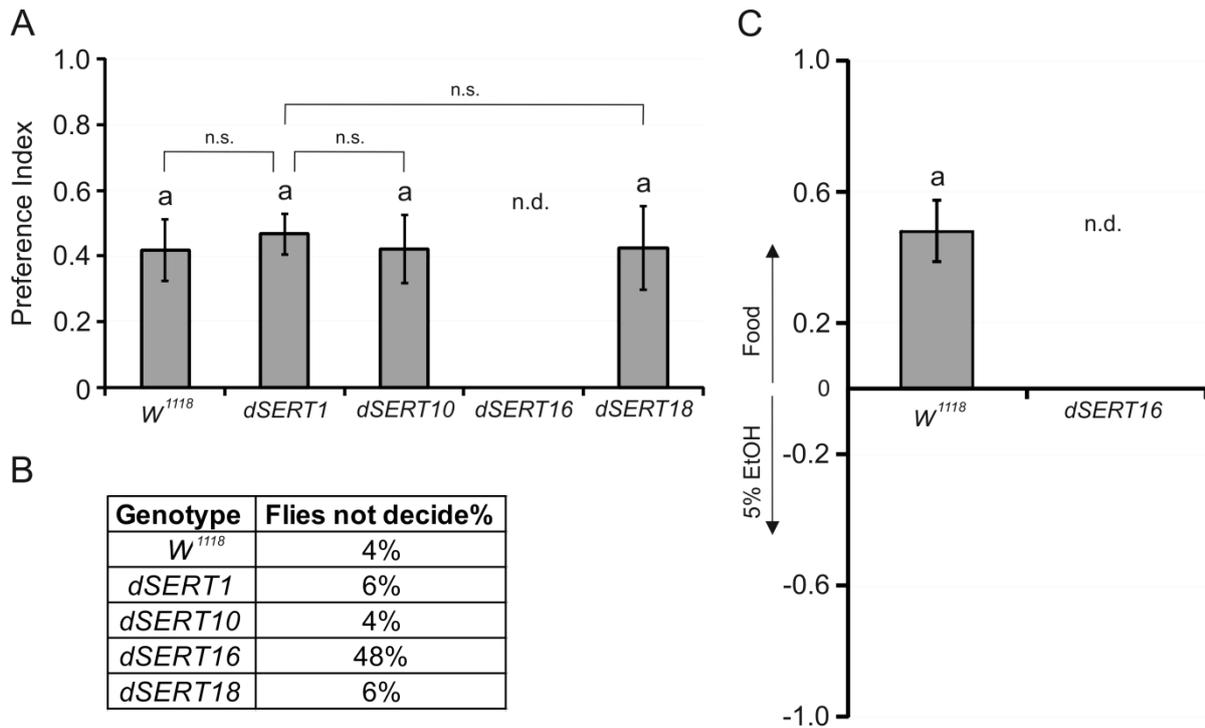
Figure 3.1.2 dSERT is required for odor preference choice. (A) *dSERT16* mutants are unable to make ethanol preference choice. *dSERT10* and *dSERT18* displayed similar ethanol preference like controls ($P > 0.05$; n=22-27). (B) Percentage of flies did not go to odor trap. 48% of total *dSERT16* failed to go into food traps. (C) Preference between food odor and 5% ethanol. W^{1118} flies prefer juice over 5% of ethanol, 40% of *dSERT16* could not go to the trap (n=12). (n.d. = no data; ANOVA posthoc test, n.s. $P > 0.05$; nonparametric one sample sign test, a= different from random)

3.1.3 The original P-element insertion line do4388 are impaired in olfactory ethanol preference and tolerance

It was showed above that lack of dSERT induced decrease of food odor initiation. To verify whether P-element insertion could influence dSERT function in ethanol preference, the original P-element insertion line for dSERT mutants' genesis do4388 was tested for ethanol preference. do4388 displayed decrease of preference to ethanol which is significantly different compared to w^{1118} and w^{1118} /do4388 (Fig. 3.1.3A). Flies with one copy of *do4388* showed normal ethanol preference like wild type. That means both *do4388* insertion sites are needed to cause an ethanol preference change. Ethanol sensitivity and tolerance was also tested to better understand *do4388* insertion site influence on dSERT function. do4388 had 19min of the MET1 which exhibit similar ethanol sensitivity with w^{1118} and w^{1118} /do4388 (P > 0.05 Fig. 3.1.3B). In contrast, do4388 only had 13% increases in MET2 which is significantly

decreased of ethanol tolerance compared to control flies (Fig. 3.1.3C). Taken together the phenotype of ethanol preference and tolerance, a conclusion could be drawn that, *do4388* insertion could depress both flies' ethanol preference and tolerance.
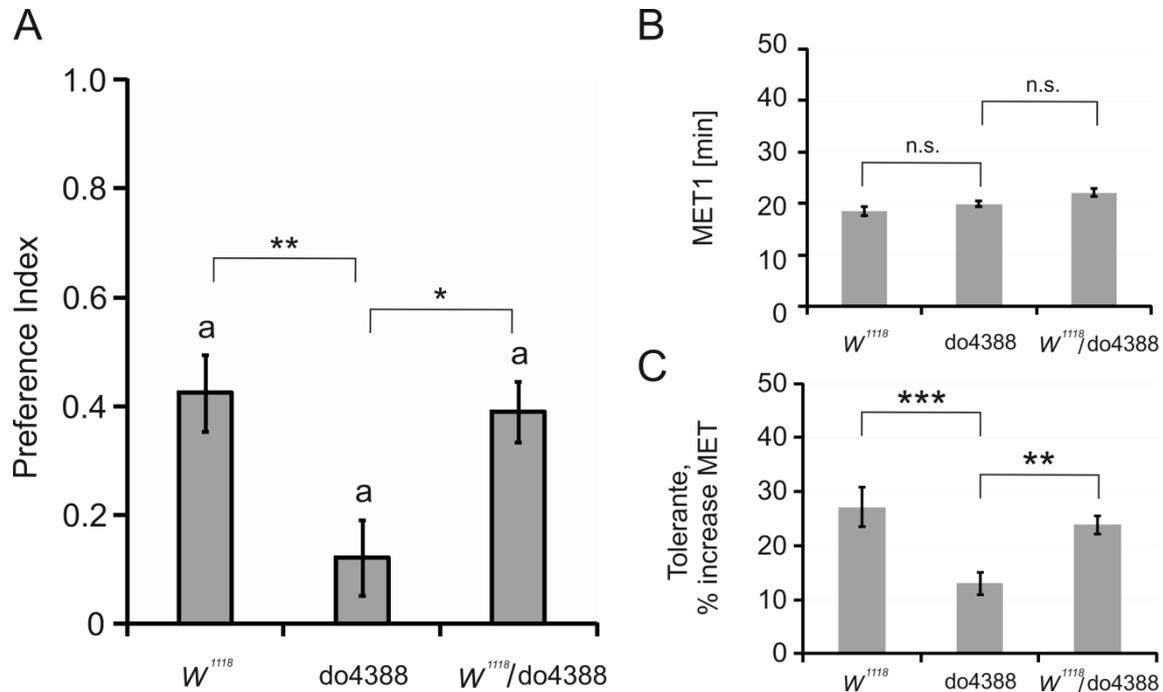

Figure 3.1.3 Original P-element insertion line do4388 has decreased ethanol preference and tolerance. (A) do4388 showed decreased preference to ethanol compared to controls ($P < 0.05$, n=34-37). (B) do4388 flies had similar MET1(19.8 ± 0.57) like w^{1118} and w^{1118}/do4388 controls. (C) Ethanol tolerance of do4388 was highly reduced in contrast with controls ($P < 0.01$, n=14). (ANOVA posthoc test, n.s. $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$; nonparametric one sample sign test, a= different from random).

3.1.4 Ethanol odour induced preference is controlled by serotonergic neurons

Flies fed with TPH precursor 5-HTP have been showed to increase the brain serotonin level (Dierich and Greenspan, 2007). After flies were treated with different concentration of 5-HTP, they were used for ethanol preference test. Flies fed with 45mM of 5-HTP lost their ethanol preference compared to those only fed with sucrose, but when fed with 5mM of 5-HTP did not affect ethanol preference (Fig. 3.1.4.1A Schläge, 2013). To get a clear idea on increase of serotonin level in which set of neurons of the brain could lead to a change in ethanol preference, UAS-*SERTDN*-GFP was generated (Ritze, 2007). In this construct the intra-cellular sites of dSERT 138 (H), 139 (R), 140 (C) were changed to Y, S, R (Fig. 3.1.4.1B).

Analysis of UAS-*SERTDN*-GFP expression in specific serotonergic driver line-*TPH*-GAL4 (Park et al., 2006) explicitly showed drop of cytoplasmic serotonin level in certain clusters (Kaiser, 2009). That suggests UAS-*SERTDN*-GFP expression suppresses extracellular 5-HT reuptake which will prolong serotonin signaling.
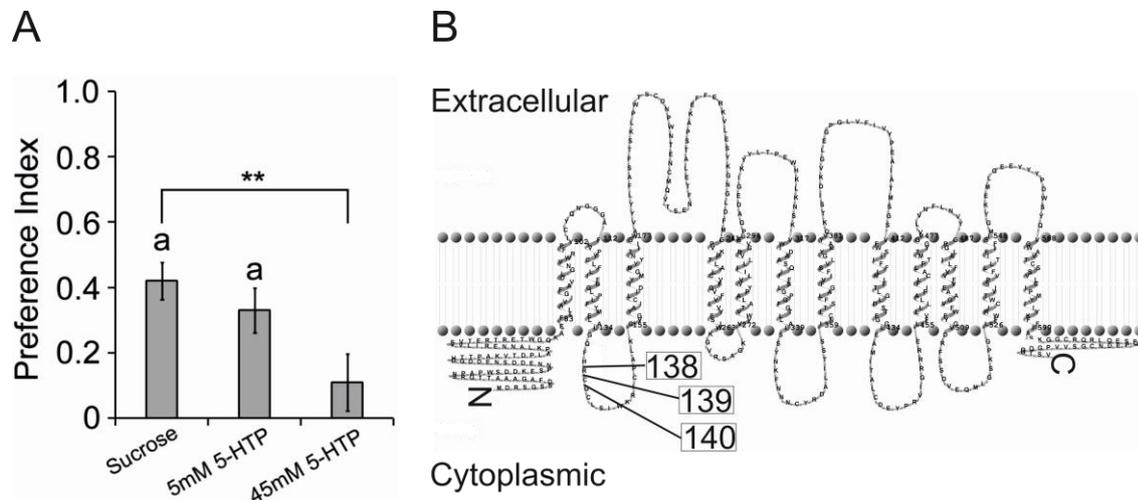

Figure 3.1.4.1 Increase serotonin level by drug feeding or genetic construct expression. (A) Increase serotonin level by fed 5-HTP decrease ethanol preference. This effect is dose dependent (Schläge, 2013). (B) Schematic drawing of UAS-*SERTDN*-GFP constructs. UAS-*SERTDN*-GFP was genetically changed from amino acid site 138,139,140 to Y, S, and R (modified after Jhamna Magsig).

Flies expressing UAS-*SERTDN*-GFP in *TPH*-GAL4 depended neurons were inserted to bilateral odor assay to measure influence of prolonged serotonin signaling in ethanol preference. UAS-*SERTDN*-GFP/*TPH*-GAL4 flies have 0.2 ± 0.05 of PI to ethanol; Control flies UAS-*SERTDN*-GFP/+ and *TPH*-GAL4/+ got 0.50 ± 0.04 and 0.49 ± 0.05 of PI respectively (Fig. 3.1.4.2A). Compared to controls, UAS-SERTDN /*TPH*-GAL4 flies' preference to ethanol was significantly reduced ($P < 0.01$). This result provides a solid evidence that serotonin modulates ethanol odor induced preference and confined the function into TPH dependent neurons. Another tool which can also increase serotonin level in flies' brain UAS-*DVMAT* was used (Chang et al., 2006) to further address that the ethanol preference decrease is due to excess serotonin in synapse. Two choice assay was performed to flies which over express DVAMT in TPH depend neurons. Compared to PI of two genetic controls UAS-*DVMT* (0.34 ± 0.05) and *TPH*-GAL4/+ (0.42 ± 0.06), UAS-*DVMT*/*TPH*-GAL4 flies' PI was 0.25 ± 0.08 which is lower (Fig. 3.1.4.2 B).
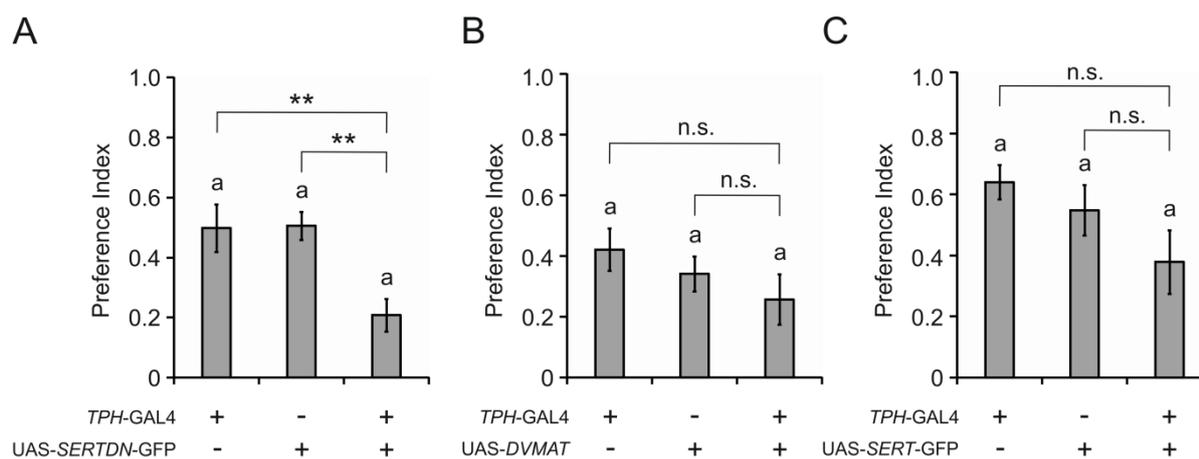
Figure 3.1.4.2 Disturbing serotonin expression in TPH dependent neurons caused reduced ethanol preference. (A) UAS-*SERTDN*-GFP/*TPH*-GAL4 had severely reduced ethanol preference (0.2±0.05) in contrast with two controls' PI ($P < 0.01$; n=28-35). (B) Over expression of DVMAT in *TPH*-GAL4 driver line did not sufficiently alter ethanol preference change from control flies (n=35-36). (C) UAS-*SERT*-GFP construct expressed in TPH depended neurons did not induce preference change (n=27-29). (ANOVA posthoc test, n.s. $P > 0.05$, ** $P < 0.01$; nonparametric one sample sign test, a= different from random)

However that difference is not significantly different from each other ($P > 0.05$). This data indicate that over expression of DVMAT in TPH neurons could not sufficiently change ethanol preference. Park et al. (2006) expressed UAS-*SERT*-GFP construct ectopically lead to 5-HT uptake. UAS-*SERT*-GFP construct was over expressed in TPH depended neurons to verify whether over load of dSERT expression will change ethanol preference in *Drosophila*. The PI of UAS-*SERT*-GFP/*TPH*-GAL4 was 0.38±0.10 which was not significantly different from two genetic controls ($P > 0.05$; Fig. 3.1.4.2C). This suggests that endogenous over expression of dSERT could not influence decision making in ethanol preference. These data indicate over expression of UAS-*SERTDN*-GFP depressing ethanol preference which could not be duplicated by over expression of UAS-*DVMT* and UAS-*SERT*-GFP construct.

To evaluate the exact serotonergic neurons that modulate ethanol preference in *TPH*-GAL4 driver line, double immunostaining of 5-HT and GFP were carried out. Both brains and thoracic ganglia ware investigated in *TPH*-AGL4/UAS-mCD8-GFP. For 5-HT staining, 12 distinct clusters were identified in the brain (Fig. 3.1.4.3A) which matched with all the clusters found before (Giang et al., 2011). GFP positive cells expressed from anterior to posterior of the brain and appeared in most of 5-HT clusters (Fig. 3.1.4.3A'). Besides expression of GFP in these 5-HT clusters there are some non-serotonergic neurons (Fig. 3.1.4.3A'' arrow). In thoracic ganglia 5-HT positive neurons (Fig. 3.1.4.3B) exist in the entire

cluster described by Vallés and White (1988). GFP positive neurons present in Pro, Meso, Meta and Abdm segment of thoracic ganglia (Fig. 3.1.4.3 B' asterisk). In the lower part of Abdm no GFP staining could be observed (Fig. 3.1.4.3B''). Next co-labeling of different serotonergic cell and GFP cells were carefully analyzed according to clusters.
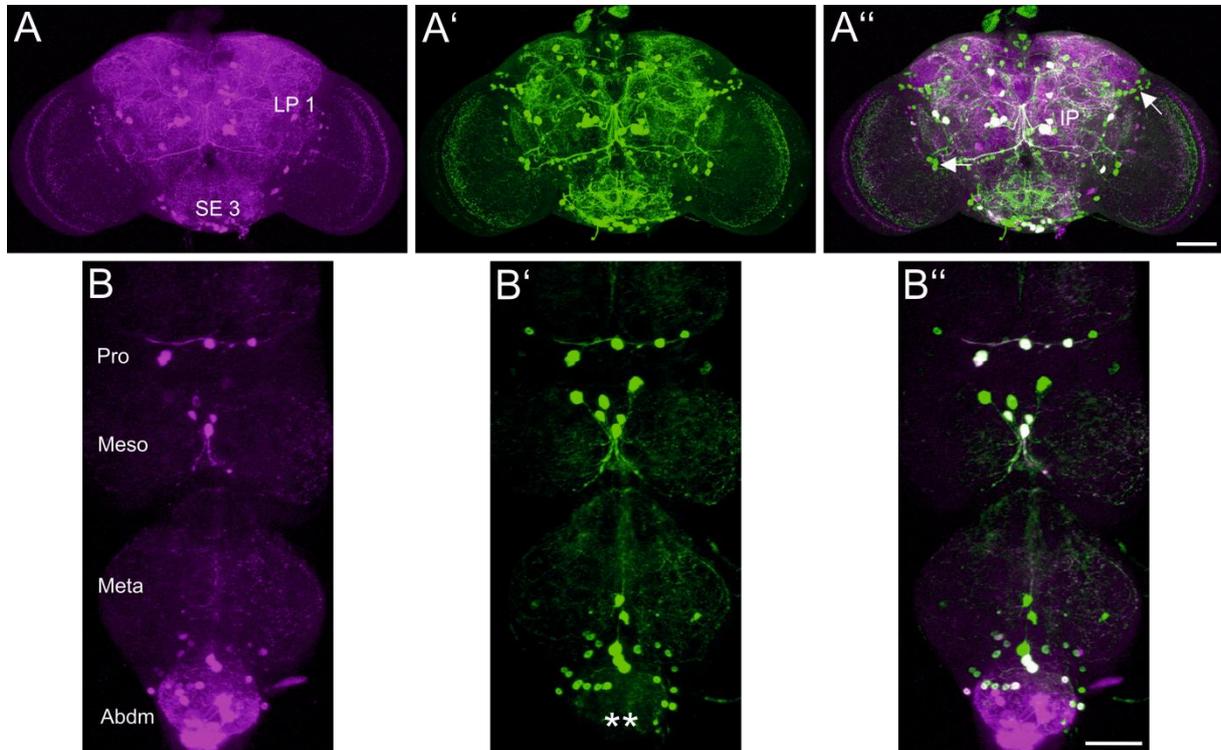

Figure 3.1.4.3 Co-localization of 5-HT and GFP in *TPH*-GAL4/UAS-mCD8-GFP flies. (A-A'') Co-localization of 5-HT and GFP in adult brain. GFP positive neurons exist in most of 5-HT clusters, non-specific serotonergic neurons were found as well. (B-B'') Overview of 5-HT and GFP overlap in adult thoracic ganglia. GFP signal could be detected through every segment in thoracic ganglia but not low part of Abdm (B' asterisk). (magenta=5-HT, green = GFP, scale bar 50μm)

The cell numbers were variable caused by dissection and staining procedure. Therefore different clusters from brains and thoracic ganglia were calculated in average. There are in total 44.2±0.3 of 5-HT positive cells and 71.3 ±0.9 of GFP positive neurons in the brain, whereas 10 clusters were found to be GFP positive out of 12 serotonergic clusters (Fig. 3.1.4.4A). Colocalization of 5-HT and GFP was found in 54 % of serotonergic cells (Table of Fig. 3.1.4.4A). Besides the 5-HT clusters TPH-GAL4 also drives expression of GFP in apparently non-serotonergic neurons. The following description about the brain was summarized in Figure 3.1.4.4A. In the lateral protocerebrum all the 5-HT positive cells of the cluster LP1 (2 cells) superimpose with GFP. Single cells as a cluster were found only in both

DP and CSD which could not be detected in GFP channel. There are three 5-HT cells found in the anterior protocerebrum (AP cluster), only the bigger one being colocalized with GFP. In

TPH-GAL4	5-HT ± STDEV	GFP ± STDEV	Merge ± STDEV	n
SP1	3.0 ± 0.0	3.7 ± 0.5	2.1 ± 0.3	21
SP2	5.2 ± 0.7	4.9 ± 1.0	2.5 ± 0.7	19
IP	7.5 ± 0.8	8.1 ± 1.3	4.6 ± 0.8	20
LP1	2.0 ± 0.0	5.4 ± 0.8	2.0 ± 0.0	14
LP2	9.8 ± 1.1	12.9 ± 1.9	2.8 ± 0.8	16
LP3	2.8 ± 0.7	16.2 ± 1.7	1.6 ± 1.0	9
SE1	4.1 ± 0.3	4.8 ± 0.9	2.2 ± 0.7	14
SE2	2.1 ± 0.3	2.1 ± 0.3	2.0 ± 0.4	13
SE3	3.0 ± 0.0	7.2 ± 0.9	3.0 ± 0.0	17
AP	2.9 ± 0.3	6.0 ± 2.4	1.0 ± 0.0	11
DP	1.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	16
CSD	1.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	17

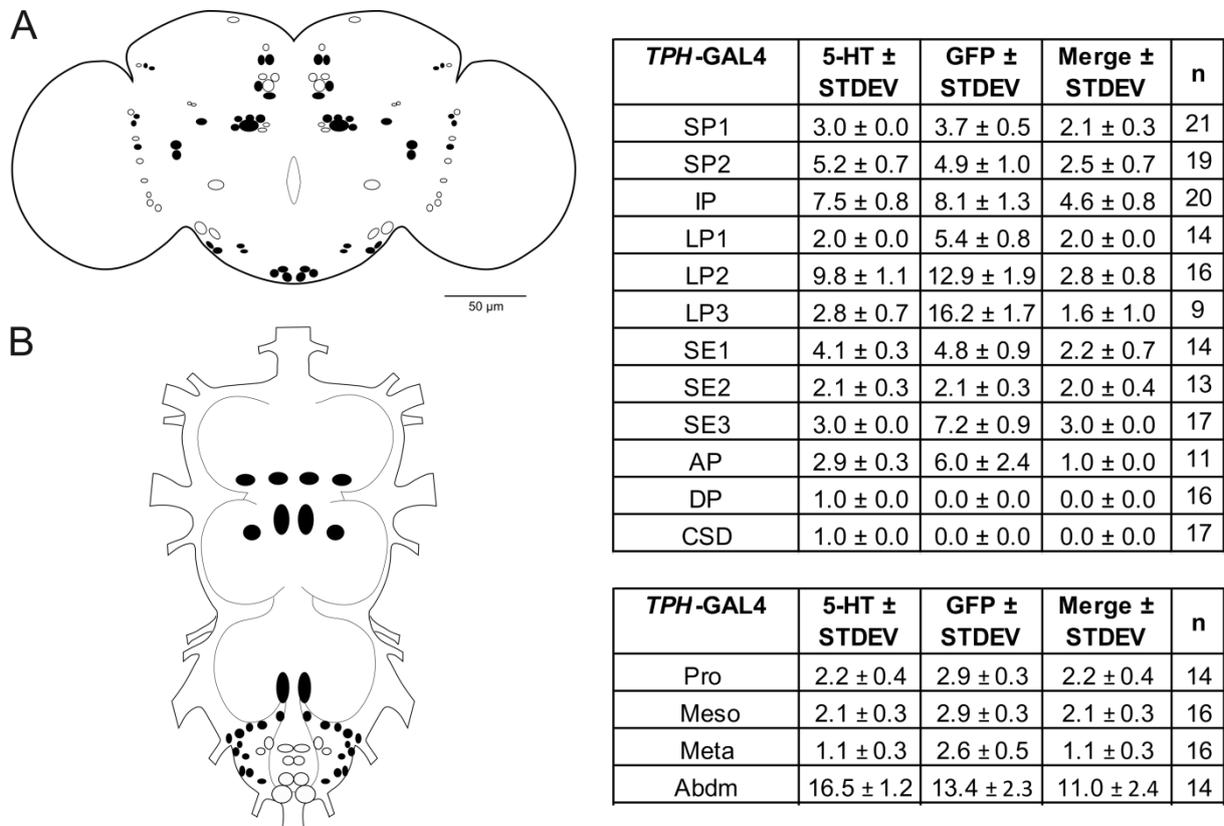| TPH-GAL4	5-HT ± STDEV	GFP ± STDEV	Merge ± STDEV	n
Pro	2.2 ± 0.4	2.9 ± 0.3	2.2 ± 0.4	14
Meso	2.1 ± 0.3	2.9 ± 0.3	2.1 ± 0.3	16
Meta	1.1 ± 0.3	2.6 ± 0.5	1.1 ± 0.3	16
Abdm	16.5 ± 1.2	13.4 ± 2.3	11.0 ± 2.4	14

Figure 3.1.4.4 Schematic drawing and summary of TPH-GAL4 expression in CNS. (A) There are 10 out of 12 serotonergic clusters co-labeling with GFP. DP and CSD cluster did not have GFP positive neurons. TPH-GAL4 could express 54% of serotonergic neuron. (B) GFP positive cells present in all the serotonergic clusters (75%) in thoracic ganglia except the lower middle part of Abdm. (circles= 5-HT positive, black dots=overlap of GFP and 5-HT, n=9-21)

the sub-esophagus region, two 5-HT cells are located in the SE2 cluster which both cells overlap with GFP. The same is true for SE3 (3 cells). In the SE1 cluster only two elliptical cells projecting back to the sub-esophagus are found to be 5-HT positive. In superior protocerebrum, two out of three cells in the SP1 cluster are both 5-HT and GFP positive. However, there is one giant round cell in SP2 only expressing 5-HT. In the IP cluster one round big cell and four small surrounding cells express both 5-HT and GFP. Details of GFP expression pattern in thoracic ganglia is shown in Figure 3.1.4.4B. In Prothoracic neuromere (Pro) six cells lined at the end of the segment, two from each hemisphere are serotonergic. In Meso cluster 4 neurons out of 6 are overlapping with 5-HT cells; somas of these neurons sit beside the middle line and project their axons to the bottom of this segment. Even though two

pairs of GFP positive cells are found at the end of Meta segment, only one pair of them was serotonergic. There are 16.5±1.2 5-HT positive cells in each side of abdominal segment which 67% are also GFP positive. GFP cells are absent in the middle part of the Abdominal segment. After knowing the details of *TPH*-GAL4 expression pattern combined with the effect of decreased ethanol preference when UAS-*SERTDN*-GFP is expressed, a conclusion can be drawn like this 54% of 5-HT positive neurons in the brain and 75% in thoracic ganglia modulate ethanol induced preference.

Results from immunostaining analysis narrow down serotonergic neuron to 54% that modulate ethanol preference. It is still ambiguous whether all the serotonergic neurons included in *TPH*-GAL4 line are unique for ethanol preference. To find out if increased serotonin level in TPH dependent neurons is sufficient to change ethanol tolerance or not, *TPH*-GAL4 was crossed to UAS-*DVMAT*-GFP then tested in inebroimeter. *TPH*-GAL4/UAS-*DVMAT*-GFP displayed 29.5±3.3 min of MET1 that is similar level as two genetic controls (Fig.3.1.4.5A). This data suggests that increased serotonin storage in TPH driven neurons could not affect ethanol sensitivity. After second round of ethanol exposure *TPH*-GAL4/UAS-*DVMAT*-GFP increased 17.0%±9.2% of MET which was not significantly different from *TPH*-GAL4/+ and UAS-*DVMAT*-GFP/+ flies (Fig.3.1.4.5 B). This indicates that more serotonin in adult brain did not alter ethanol tolerance.
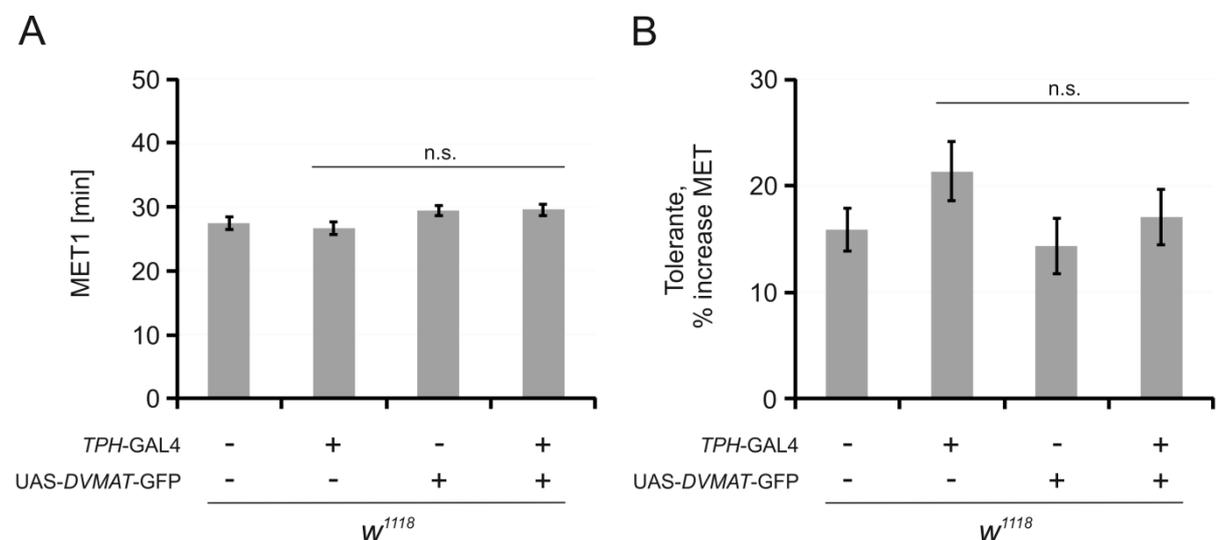

Figure 3.1.4.5 Increased serotonin storage in TPH manner neurons did not change ethanol sensitivity and tolerance. (A) *TPH*-GAL4/UAS-*DVMAT*-GFP showed similar ethanol sensitivity as control flies. (B) Increased serotonin storage by expressing DVMAT did not change ethanol tolerance. (ANOVA posthoc test, n.s. $P > 0.05$, n=13).

3.1.5 Serotonin transporter present in the same neurons with serotonin in adult *Drosophila* brain but not in abdominal ganglia

It has already been shown that TPH positive neurons are located in the same cells of serotonergic neuron in *Drosophila* adult brain (Bao et al., 2010). In larval brain, dSERT exclusively expressed in the serotonergic neurons and same result also found in adult olfactory pathway (Giang et al., 2011). However, data of the precise analysis of dSERT positive neurons and colocalization with 5-HT in adult CNS was missing. *TPH*-GAL4 can drive about 54% of serotonergic neurons in adult brain, and it has also been characterized with serotonin overlapping pattern. To investigate the expression pattern of dSERT in *TPH*-GAL4 driven brains, crosses of *TPH*-GAL4 and UAS-mCD8-GFP were stained and analyzed. SERT staining could be visualized in anterior, medium and posterior of adult brain. In anterior part, SERT positive neurons cluster like LP2, SE1, CSD and AP cluster could be observed, as well as some SERT positive fibers in AL and LP (Fig. 3.1.5.1A). GFP staining exists in most of the SERT positive clusters but not CSD neurons and two big cells form SE1 (Fig. 3.1.5.1A'').
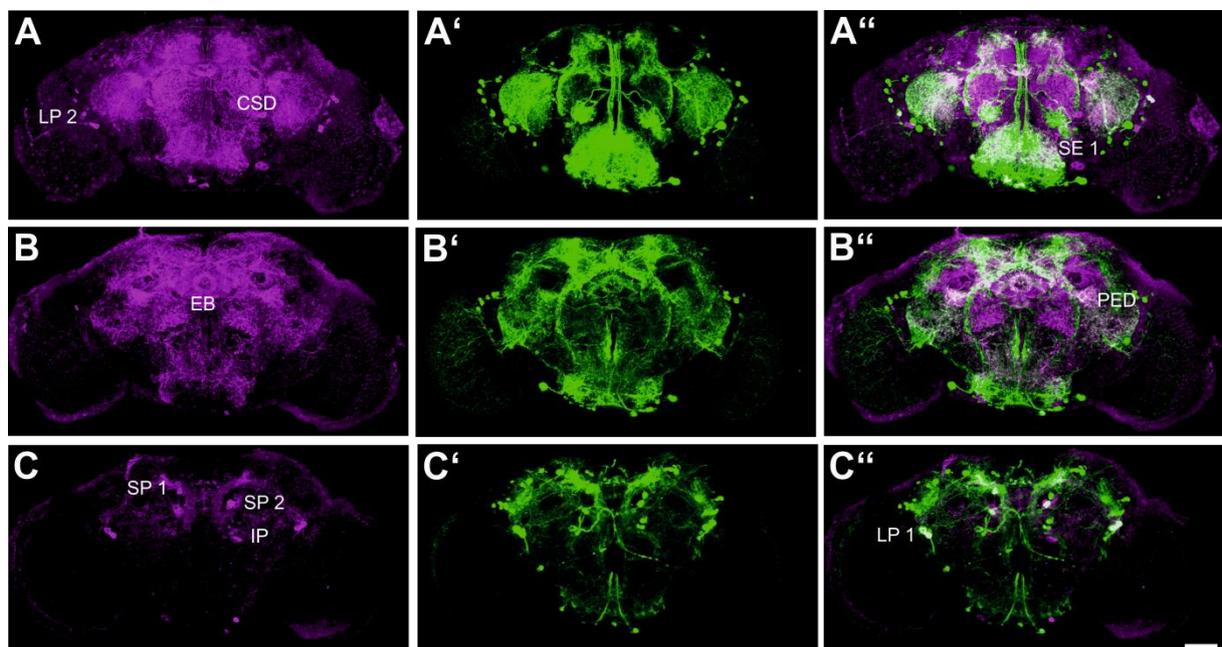

Figure 3.1.5.1 Co-expression of GFP and dSERT in TPH dependent neurons. (A-A'') Anterior co-labeling of dSERT and GFP. dSERT positive cells appeared in LP2, CSD and subesophageal ganglia (A), but not all these neurons were overlapping with GFP. (B-B'') Medium section of the brain stained with dSERT with GFP. Both GFP and dSERT signal could be detected in EB, FB and FED. (C-C'') LP1, SP1, SP2 and IP clusters were found in posterior of adult brain. (magenta=dSERT, green = GFP, scale bar 50μm)

In medium section of dSERT staining, SERT is abundantly present in EB, FB and pedunculus (PED) (Fig. 3.1.5.1B) which clearly showed co-labeling with GFP signal (Fig. 3.1.5.1B''). Two cells in LP1 cluster and SP1, SP2, IP cluster were found in the posterior part of the brain (Fig. 3.1.5.1A). The merge signal from both 5-HT and GFP could clearly be seen in posterior of brain (Fig. 3.1.5.1A'').

To quantify dSERT expression in brains, different clusters with dSERT and GFP staining were analyzed. 11 clusters were found for dSERT positive neurons and all of them being identical to the 5-HT clusters described above. There are in total 36 dSERT positive cells in all clusters; 19 of them also being GFP positive (Figure 3.1.5.2D). In adult brain, cells positive for dSERT are almost identical with the 5-HT staining. LP1 cluster which is known as two serotonergic neurons that colabel with GFP in *TPH*-GAL4/UAS-mCD8-GFP brain had the same expression of dSERT (Figure 3.1.5.2A). SE3 cluster have three serotonergic neurons at each side of the low suboesophageal; the same is true for dSERT staining (Figure 3.1.5.2B). Some other serotonergic clusters which not all were GFP positive, for example in SP1 cluster one cell express dSERT but miss in GFP channel (Figure 3.1.5.2 C' asterisk).The LP3 cluster was found likely to be dSERT positive.

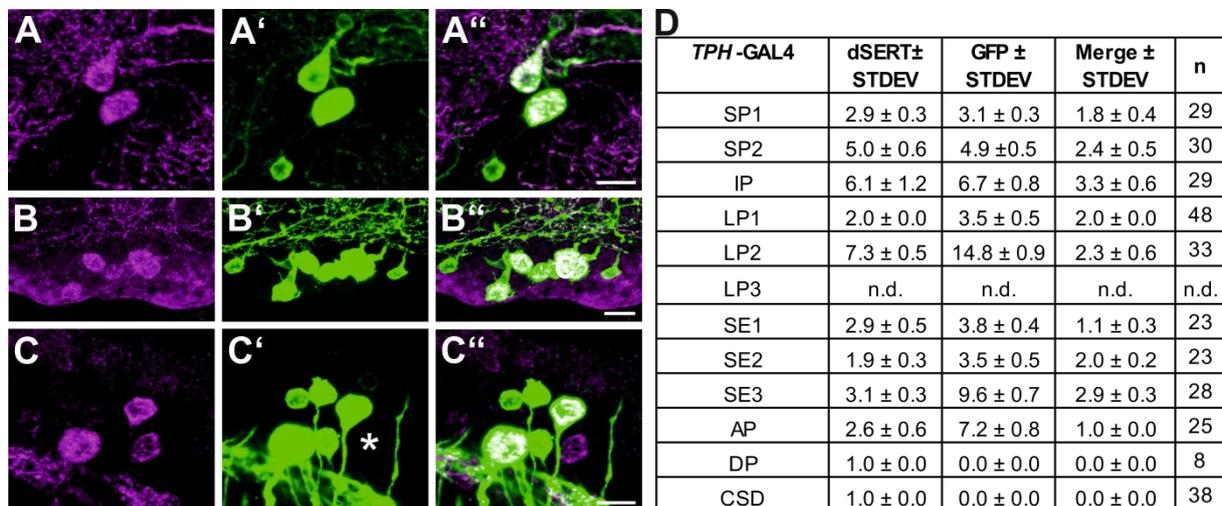| *TPH*-GAL4	dSERT± STDEV	GFP ± STDEV	Merge ± STDEV	n
SP1	2.9 ± 0.3	3.1 ± 0.3	1.8 ± 0.4	29
SP2	5.0 ± 0.6	4.9 ±0.5	2.4 ± 0.5	30
IP	6.1 ± 1.2	6.7 ± 0.8	3.3 ± 0.6	29
LP1	2.0 ± 0.0	3.5 ± 0.5	2.0 ± 0.0	48
LP2	7.3 ± 0.5	14.8 ± 0.9	2.3 ± 0.6	33
LP3	n.d.	n.d.	n.d.	n.d.
SE1	2.9 ± 0.5	3.8 ± 0.4	1.1 ± 0.3	23
SE2	1.9 ± 0.3	3.5 ± 0.5	2.0 ± 0.2	23
SE3	3.1 ± 0.3	9.6 ± 0.7	2.9 ± 0.3	28
AP	2.6 ± 0.6	7.2 ± 0.8	1.0 ± 0.0	25
DP	1.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	8
CSD	1.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	38

Figure 3.1.5.2 Details of dSERT positive neurons overlap within TPH dependent neurons. (A-A'') Two LP1 cells were dSERT positive and overlapping with GFP. (B-B'') All the cells in SE3 cluster were also express dSERT. (C-C'') Three dSERT positive neurons in SP1 cluster, two of them were also GFP positive. (D) Summary of dSERT and GFP positive neurons in *TPH*-GAL4/UAS-mCD8-GFP brain. There are 36 dSERT positive neurons in adult brain; *TPH*-GAL4 could drive 53% of them to express GFP. (magenta = dSERT, green = GFP scale bar 10 μm)

Due to the weak staining and unspecific signals the analysis of SE1 for colocalization turned out to be inconclusive. DP and CSD neuron which found to be single neurons in serotonin staining were also present in dSERT staining (Figure 3.1.5.2 D). In short, in adult brain the colocalization pattern of dSERT and GFP is also the same as for 5-HT and GFP. This finding confirms the idea that all the serotonergic neurons in the adult brain are dSERT positive as well.

Thoracic ganglia kept most of its neurons from larval stage (Truman and Bate 1988). To verify all the dSERT positive neuron in adult stage are still seroronergic in thoracic ganglion, *TPH*-GAL4flies which already been analyzed for 5-HT expression were crossed again to UAS-mCD8-GFP. Adult thoracic ganglia were dissected then stained with dSERT and GFP. dSERT positive cells were found in Pro and Meso segment of thoracic ganglia, they are also colocalized with GFP (Fig. 3.1.5.3 A"). Beside neurons which were both dSERT and GFP positive, there were also some small round dSERT staining present on the surface of each segment (Fig. 3.1.5.3B arrow). At the top of the abdominal ganglia small round dSERT neurons colabelling with GFP on each side, but in the central lower part no dSERT neuron could be found (Fig. 3.1.5.3B'' arrow head).

Average of dSERT and GFP positive neurons in each cluster of thoracic ganglia was listed in Figure 3.1.5.3C. In Pro, two cells were with dSERT staining that include in the GFP cells. There was also 100% of colocalization of GFP with dSERT positive neurons in Meso and Meta. However, only 9 neurons in Abdm were expressing dSERT and 7 of them also express GFP.
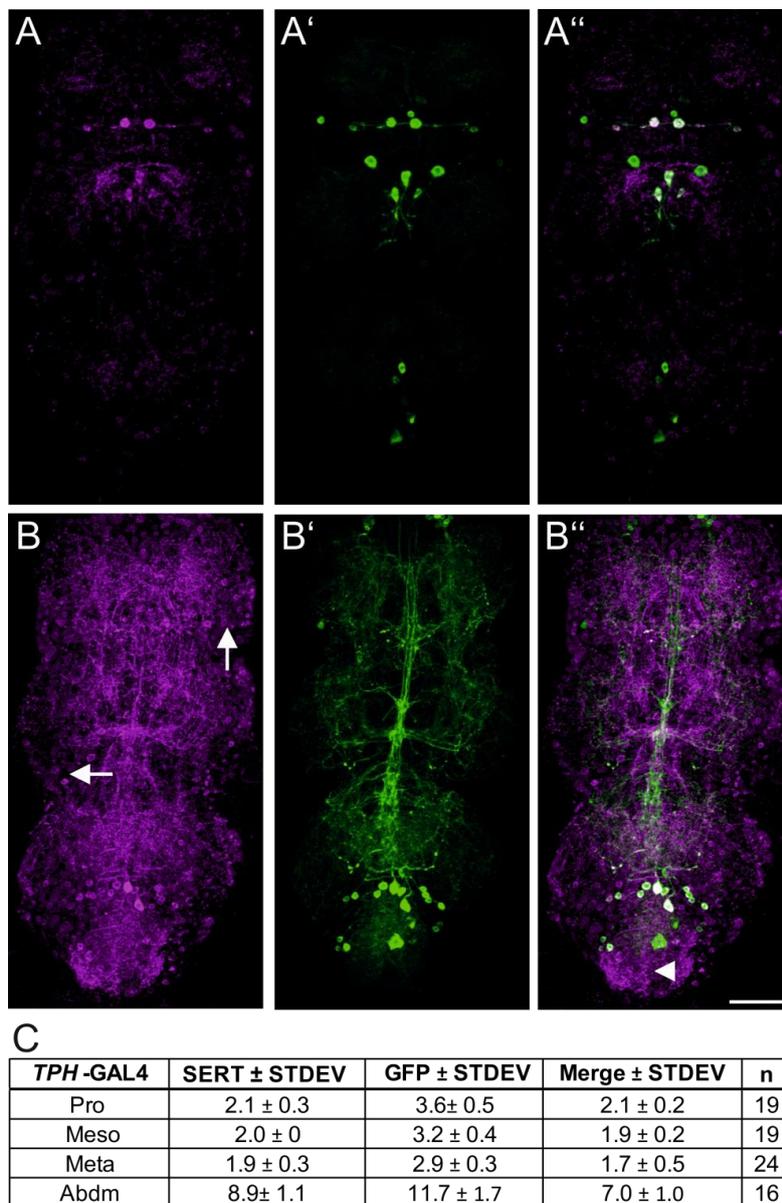
TPH-GAL4	SERT ± STDEV	GFP ± STDEV	Merge ± STDEV	n
Pro	2.1 ± 0.3	3.6± 0.5	2.1 ± 0.2	19
Meso	2.0 ± 0	3.2 ± 0.4	1.9 ± 0.2	19
Meta	1.9 ± 0.3	2.9 ± 0.3	1.7 ± 0.5	24
Abdm	8.9± 1.1	11.7 ± 1.7	7.0 ± 1.0	16

Figure 3.1.5.3 Thoracic ganglia expression of GFP and dSERT in TPH dependent neurons. (A-A") Anterior of thoracic ganglia. dSERT expressed in Pro and Meso and that all over lapped with GFP. (B-B") Some small round dSERT positive cells which were not belonging to any serotonergic cluster located on the surface of thoracic ganglia (arrow head). dSERT positive cells could not be found in the end of Abdm where usually will be a serotonergic cluster (arrow head). (C) Average cell number of dSERT and GFP in each cluster of thoracic ganglia. (maganta = dSERT, green = GFP, scale bar 50μm)
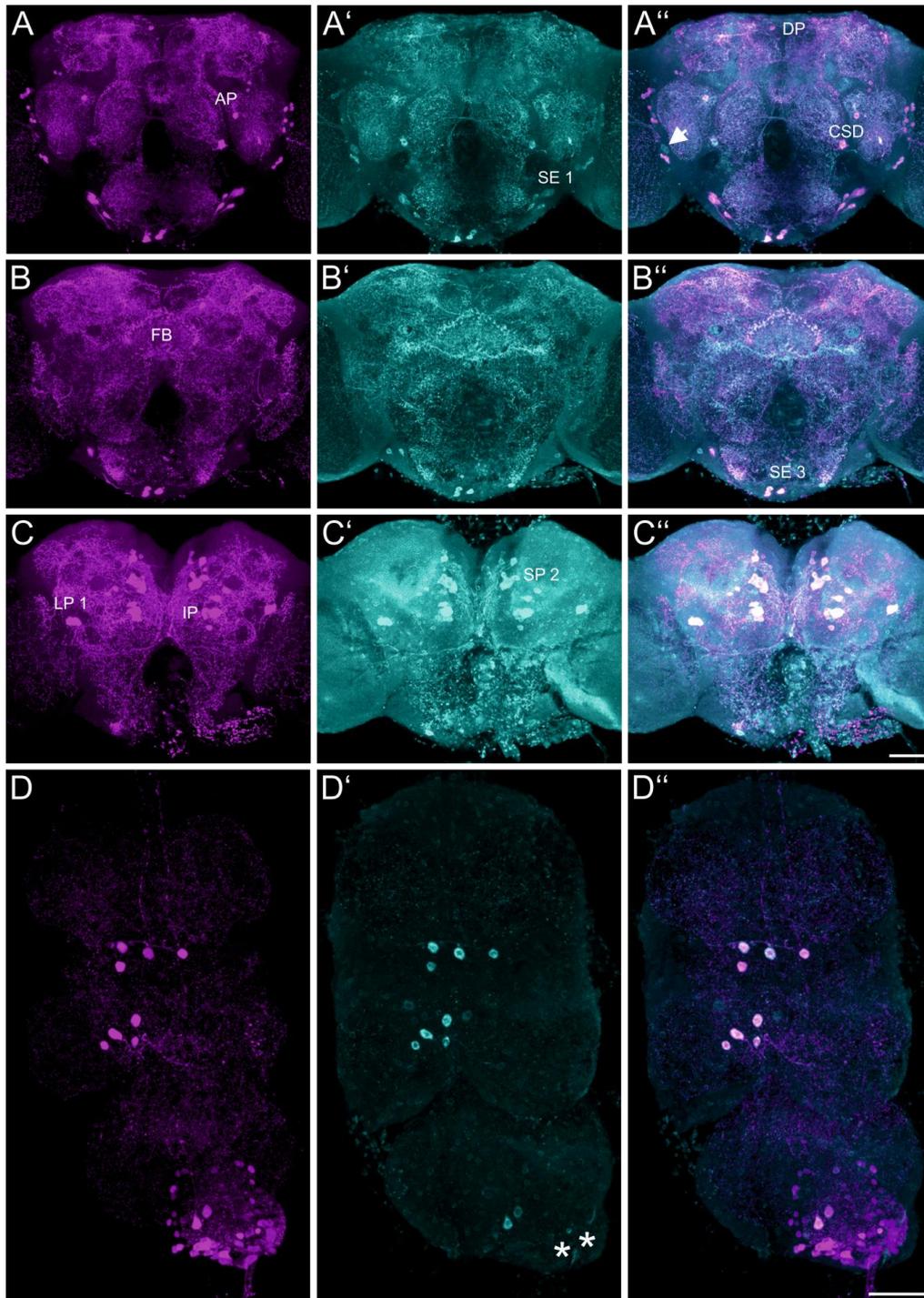
Figure 3.1.5.4 Co-expression of serotonin and dSERT in adult CNS. (A-A'') Anterior section of dSERT and 5-HT over lapping pattern. AP, DP, CSD, LP2 and SE1 clusters could be seen in both channels. Most cells in each clusters were over lapped, except two adjacent dSERT positive cells in LP2 cluster (A'' arrow head). (B-B'') Medium section of dSERT and 5-HT expression. In FB and SE3 neurons express both 5-HT and dSERT. (C-C'') Posterior section of dSERT and 5-HT expression. Neurons in LP1, SP1, SP2 and IP clusters were both 5-HT and dSERT positive. (D-D'') dSERT and 5-HT expressed in thoracic ganglia. In Pro, Meso, Meta segment 5-HT and dSERT were well colocalized. In down part if Abdm dSERT signal could not be detect (D' asterisk) only 5-HT positive neurons were visualized. (magenta= 5-HT, cyan= dSERT, scale bar 50μm)

TPH-GAL4 line was used to driver expression of UAS-mCD8-GFP then 5-HT and dSERT expression pattern was analyzed with GFP in adult CNS separately. Even though there was some variation between dSERT and 5-HT positive clusters, the exact reason is still unclear. To identify whether difference between dSERT and 5-HT positive cells is due to staining procedure, double staining of dSERT and 5-HT were carried out. In anterior of the brain, serotonin signal was found in AP, DP, LP2, SE1 and CSD cluster as well as dSERT signal (Fig. 3.1.5.4 A, A'). Two dSERT positive cells located in lower part of LP2 cluster were not 5-HT positive (Fig. 3.1.5.4 A''). In the medium section of the brain, serotonin and dSERT staining very well present in central complex; SE3 cluster with colocalization of dSERT and 5-HT were also observed (Fig. 3.1.5.4B). In posterior of the brain, all the serotonergic neuron in LP1, SP1, SP2 and IP cluster were also dSERT positive (Fig. 3.1.5.4C). Serotonergic neurons over lapping with dSERT positive neurons in Pro, Meso and Meta segment of thoracic ganglia. In the lower part of Abdm segment big round 5-HT positive neurons clustered together then send the projection down to the end, but these neurons could not be seen in dSERT channel (Fig. 3.1.5.4D). The results from the 5-HT and dSERT antibody staining analysis suggest that serotonergic neurons in the brain are also dSERT positive, but not all the serotonergic neurons in thoracic ganglia could express dSERT.
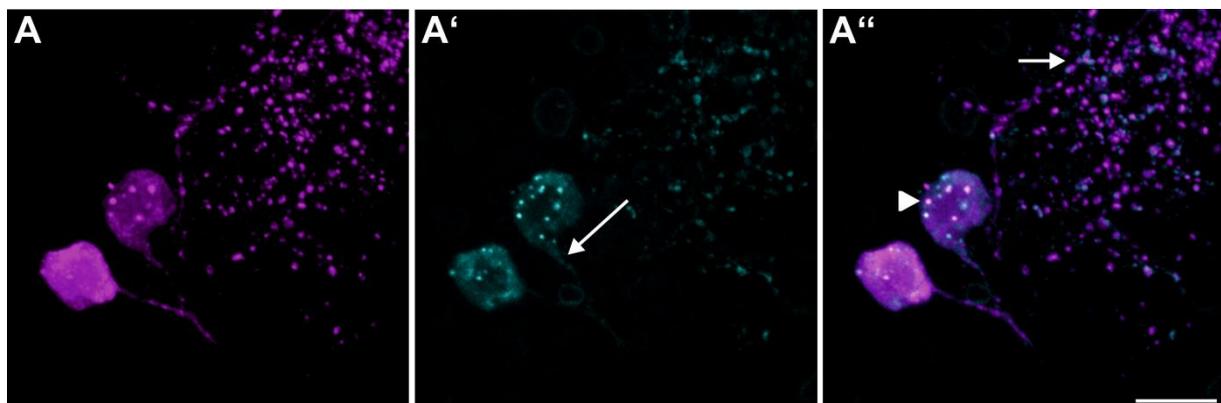

Figure 3.1.5.5 dSERT distributed majorly on the surface of serotonergic neurons. (A) 5-HT staining of SE2 cluster. 5-HT present in the cells body and axons. (A') dSERT staining of SE2 cluster. dSERT stay in the cell surface and axon (arrow). (A'') Over lapping of dSERT. Vesicles of dSERT and 5-HT colocalized at the surrounding of the cells (arrow head). Serotonin and dSERT signal next to each other in synaptic boutons (arrow). (magenta= 5-HT, cyan= dSERT, scale bar 10μm)

As the result found before, in adult brain serotonergic neurons there are dSERT present. To better understand how dSERT is distributed in serotonergic neurons, SE2 cluster which

express both 5-HT and dSERT was chosen to be scanned in higher magnification. In SE2 cluster serotonin was spread all over the cell body as well as the axons projecting down (Fig. 3.1.5.5 A). dSERT in SE2 cluster mainly stayed in the surrounding of the cell body that leave an empty hole in the center of the cell; besides dSERT could also be found in axon (Fig. 3.1.5.5 A' arrow). There were some vesicles surrounding the cell body in both dSERT and 5-HT staining, they were over lapping with each other when merge two channels together (Fig. 3.1.5.5 A'' arrow head). In synaptic boutons dSERT signal and 5-HT signal next to each other (Fig. 3.1.5.5 A'' arrow). Suggesting that 5-HT present in the cell body, dSERT located in the membrane; 5-HT was released to the synapse cliff and dSERT stay in the pre-synapse.

3.1.6 Ethanol odour induced preference is controlled by two pairs of serotonergic neurons in the brain

TPH depended neurons could lead to a decreased ethanol preference. Immuno-staining analysis narrows the serotonergic neurons down to 24 in the brain and 17 in the thoracic ganglia that mediates ethanol preference. However, it is not so clear which is the exact set of neurons responsible for the behaviour change. To further dig out the specific neuron controlling odor induced behavior, *SERT3*-GAL4 (Herb, 2005) was crossed with UAS-*SERTDN*-GFP then tested for ethanol preference. Flies express both *SERT3*-GAL4 and UAS-*SERTDN*-GFP showed 0.22 ± 0.02 of PI which is significantly decreased from control flies (Fig. 3.1.6.1, Gräber, 2012). This indicates that disturbing dSERT function in *SERT3*-GAL4 depended neurons could induce reduction in ethanol preference. Since same phenotype was found in *TPH*-GAL4/ UAS-*SERTDN*-GFP flies, the common serotonergic neurons found in *SERT3*-GAL4 should be the one that controls ethanol preference.
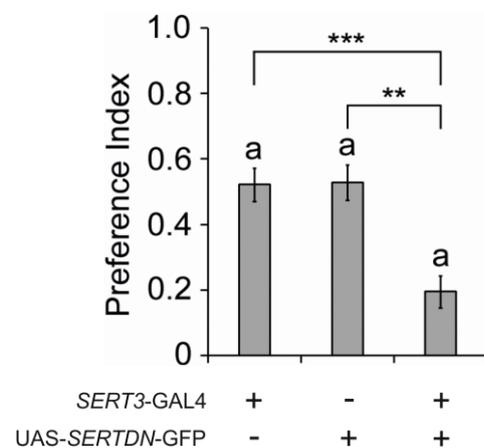

Figure 3.1.6.1 Disturbed dSERT expression in SERT3 dependent neurons decreases ethanol preference. SERT3-GAL4/UAS-*SERT*-GFP flies have PI of 0.22 ± 0.02, that was severe reduction compare to its controls. (ANOVA posthoc test, *** $P < 0.001$, ** $P < 0.01$; nonparametric one sample sign test, a= different from random, n=15-27, Gräber, 2012)

To identify which set of neuron is mediating ethanol preference *SERT3*-GAL4 flies were crossed to UAS-mCD8-GFP then stained against GFP and 5-HT. Firstly thoracic ganglia were analysed. 5-HT staining was found all over the thoracic ganglia, in Pro, Meso, Meta and Abdm segment serotonergic cells doted beside the middle line (Fig. 3.1.6.2A).
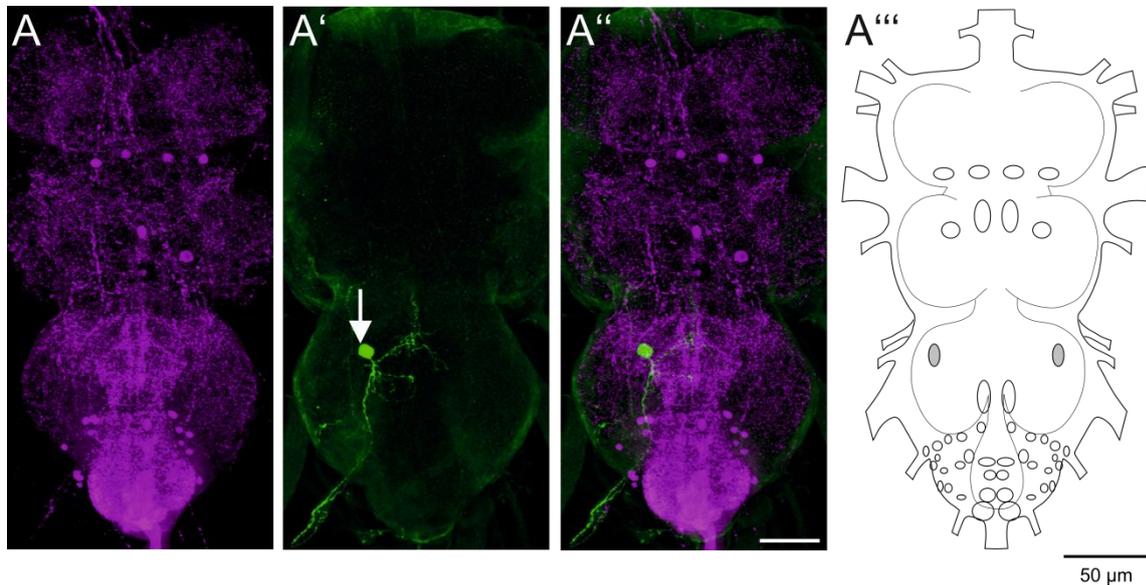

Figure 3.1.6.2 No overlap of serotonin and GFP in the thoracic ganglia of SERT3-GAL4 driver line. (A) Serotonin signal present trough the whole thoracic and abdominal ganglia. (A') GFP positive neurons were found in Meta segment. (A") Merge channel of 5-HT and GFP. The GFP positive neurons were not serotonin positive. (A''') Schematic drawing of 5-HT and *SERT3*-Gal4 driven neurons. (magenta=5-HT, green=GFP, scale bar 50μm, n=10-16)

In posterior of the thoracic ganglia, there were two paired GFP positive cells which sent projection to middle line of Meta (Fig. 3.1.6.2A'). When merge GFP signal with 5-HT signal there was no overlap (Fig. 3.1.6.2A"). More thoracic ganglia ware analyzed (n=10-16) to confirm there was no GFP positive cells expressing 5-HT and showed in the schematic drawing (Fig. 3.1.6.2A'''). This suggests that neurons modulating ethanol preference were not in the thoracic ganglia. *SERT3*-GAL4 cannot drive any serotonergic neuron in GFP expression in thoracic ganglia; therefore the preference is controlled by neurons in the brain. To identify the serotonergic neurons in adult brain that controlling ethanol induced preference, adult brain was analyzed.
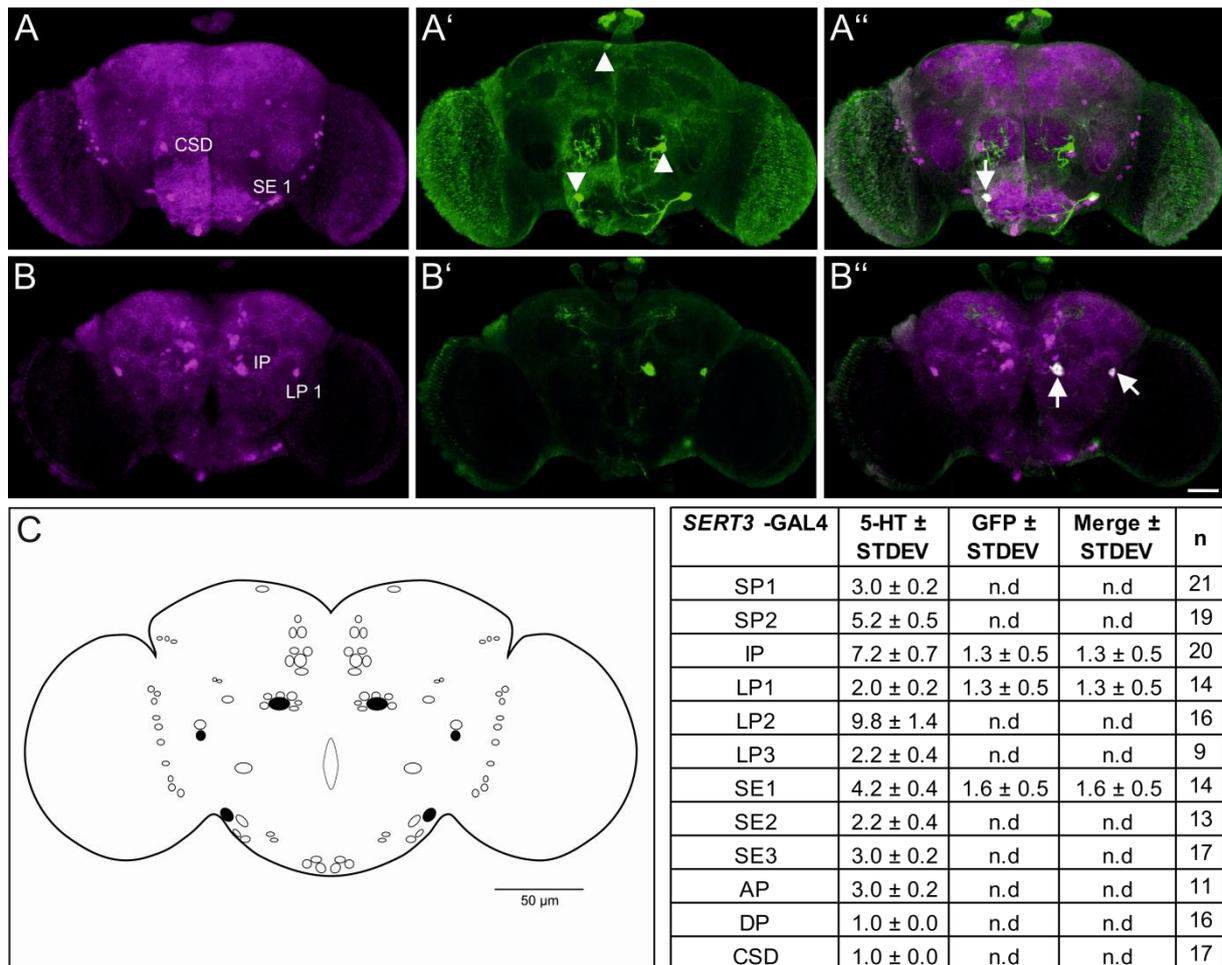
Figure 3.1.6.3 Schematic drawing of *SERT3*-GAL4 expression pattern in brain. (A-A'') Three different GFP positive clusters were detected in anterior of adult brain (arrow head). Only the cell in SE1 cluster was expressing both GFP and serotonin. (B-B'') Two distinct GFP cluster were found in posterior of adult brain. Both of that GFP cells were also 5-HT positive (arrow). (C) There are three serotonergic cells driven by *SERT3*-GAL4 in each hemisphere which is IP, LP1 and SE1 cluster. (magenta=5-HT, green=GFP, circles= 5-HT positive, black dots=overlap of GFP and 5-HT, n=9-21, scale bar 50μm).

In anterior of the brain, serotonin positive neurons in CSD, SE1, LP2 and AP clusters were clearly seen (Fig. 3.1.6.3A). There were three clusters found in anterior brain of GFP channel, one cluster on top of the brain, another on the surface of antennal lobe and the third one near SOG (Fig. 3.1.6.3A' arrow head). After merge two channel together, both 5-HT and GFP signal were detected in SE1 cluster (Fig. 3.1.6.3A'' arrow), but another two GFP positive clusters did not express 5-HT (Fig. 3.1.6.3A''). In posterior of the brain, serotonergic cells were found in SP1, SP2, IP and LP1 clusters (Fig. 3.1.6.3B); two distinct clusters of GFP positive neurons were also detected (Fig. 3.1.6.3B'). Colocalization figure showed that two GFP positive cells were also 5-HT positive and they belong to IP and LP1 cluster separately

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(Fig. 3.1.6.3 B''). Since GFP expression was variable from different brains, more brains were analyzed to get accurate colocalization information. *SERT3*-GAL4 can drive three clusters of serotonergic neurons which are LP1, IP and SE1 cluster (Fig. 3.1.6.3C). Beside the three clusters described, in two of the brains there was also one cell in SP2 cluster that was observed. Because of the rear cases, only the stable neurons count in to analysis. For IP cluster there was one giant GFP positive cell which was serotonin positive, in SE1 cluster there was an asteroid cell which is also serotonergic, one LP1 cell was found to be 5-HT positive (Fig. 3.1.6.3 Table). In summary, SERT3-GAL4 could drive about six serotonergic neurons in adult CNS of *Drosophila*.

Disturbing dSERT function by expressing UAS-*SERTDN* in *TPH*-GAL4 and *SERT3*-GAL4 dependent neurons reduced ethanol preference in both cases. Neuro-anatomical analyses revealed that 10 clusters from *TPH*-GAL4 and three clusters from *SERT3*-GAL4 were serotonergic. Thus the common serotonin positive neurons in both driver lines induced ethanol preference change. To point out the ethanol mediating neuron, details of TPH, LP1 and IP clusters in both driver lines were analyzed in higher magnification.
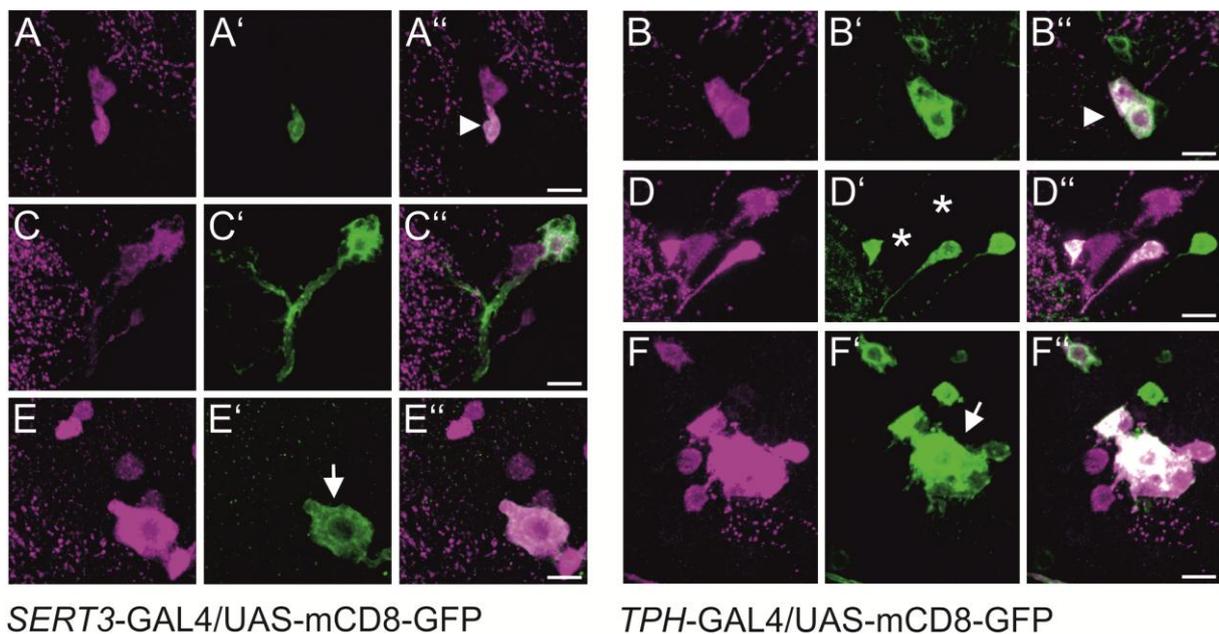

Figure 3.1.6.4 Comparison of LP1, SE1 and IP clusters from *SERT3*-GAL4 and *TPH*-GAL4. (A-A'') One neuron from LP1 cluster expressing both GFP and SERT (arrow head). (B-B'') Two LP1 cells were covered by GFP signal (arrow head). (C-C'') One GFP positive cell in SE1 expresses serotonin. (D-D'') Two big cells in SE1 cluster did not colocalized with GFP signal (asterisk). (E-E'') One big cell from IP cluster was serotonergic. (F-F'') Most of serotonin positive cells in IP cluster were also GFP positive includes the big cell (arrow). (maganta=5-HT, green=GFP, scale bar 10μm)

In LP1 cluster there are two serotonergic neurons; one of them was co-expressed with GFP (Fig. 3.1.6.4A arrow head) in *SERT3*-GAL4/UAS-mCD8-GFP, both of them were GFP positive in *TPH*-GAL4/ UAS-mCD8-GFP (Fig. 3.1.6.4B arrow head). In SE1 cluster of *SERT3*-GAL4/UAS-mCD8-GFP, only one big asteroid GFP positive neuron was found which was also 5-HT positive (Fig. 3.1.6.4C). The same asteroid cell was observed in 5-HT staining of *TPH*-GAL4/UAS-mCD8-GFP SE1 cluster, however this cell did not appear in GFP staining (Fig. 3.1.6.4D asterisk). In the merge channel of *TPH*-GAL4 SE1 cluster, only two small elongated GFP cells merged with 5-HT (Fig. 3.1.6.4D'') which indicated the absence of same SE1 cell as *SERT3*-GAL4. There was only one giant GFP positive neuron over lapping with 5-HT in IP cluster of *SERT3*-GAL4/UAS-mCD8-GFP (Fig. 3.1.6.4E arrow), rest smaller cells from IP cluster did not express GFP signal. In *TPH*-GAL4/UAS-mCD8-GFP, most of the serotonergic cells overlap with GFP in IP cluster including the big one in the center (Fig. 3.1.6.4F arrow). The common cells which expressed in *SERT3*-GAL4 and *TPH*-GAL4 are one cell in LP1 cluster and one cell in IP cluster. This result addressed that one big serotonergic neuron from IP cluster and another neuron from LP1 controls ethanol preference.

With comparison of common neurons in *TPH*-GAL4 and *SERT3*-GAL4, one neuron in IP cluster and one in LP cluster were found to be the potential neurons that determines ethanol preference. Neuron position and projection is required for the formation of neuronal circuits (Karim and Moore, 2011). To get more interpretation about what would be every neurons function in *SERT3*-GAL4 driver line, *SERT3*-GAL4 was crossed to UAS-mCD8-GFP. Brains from *SERT3*-GAL4/UAS-mCD8-GFP flies were dissected and stained with GFP and nc82 (recognize synapse active zone). In IP cluster, there was one big neuron located on each side of the brain; it sends its axon to the middle line of the brain then three branches are formed. One of the branch stretched following inner antenna-cerebral tract to lateral horn, another one reach to calyx then surround it by small fibers, the third one firstly follow middle line then turn to lobula (Fig. 3.1.6.5A). The asteroid neuron on the surface of SOG belongs to SE1 cluster. It stretched its axon to the SOG then split one branch to arborize in the upper middle part of SOG and another one to thoracic ganglia (Fig. 3.1.6.5B arrow). The LP1 soma appeared at the posterior of the brain, it then project through the brain and merged to ventrolateral protocerebrum (Fig. 3.1.6.5C). When using Brainbow system to get single neuron projection from *TPH*-GAL4, the same pattern was observed in LP1 cluster (Fig. 3.1.6.5C'). To rule out the interference from non-serotonergic neurons in *SERT3*-GAL4,

projection pattern of those neurons were also analyzed. A cluster of neurons stayed on top of the brain middle line and sent axons until inside border of AL ((Fig. 3.1.6.5D, arrow head). On surface of AL few cells stick together; they sent one branch to AL and another one crossed AL then arborized in superior lateral protocerebrum (Fig. 3.1.6.5D, arrow).
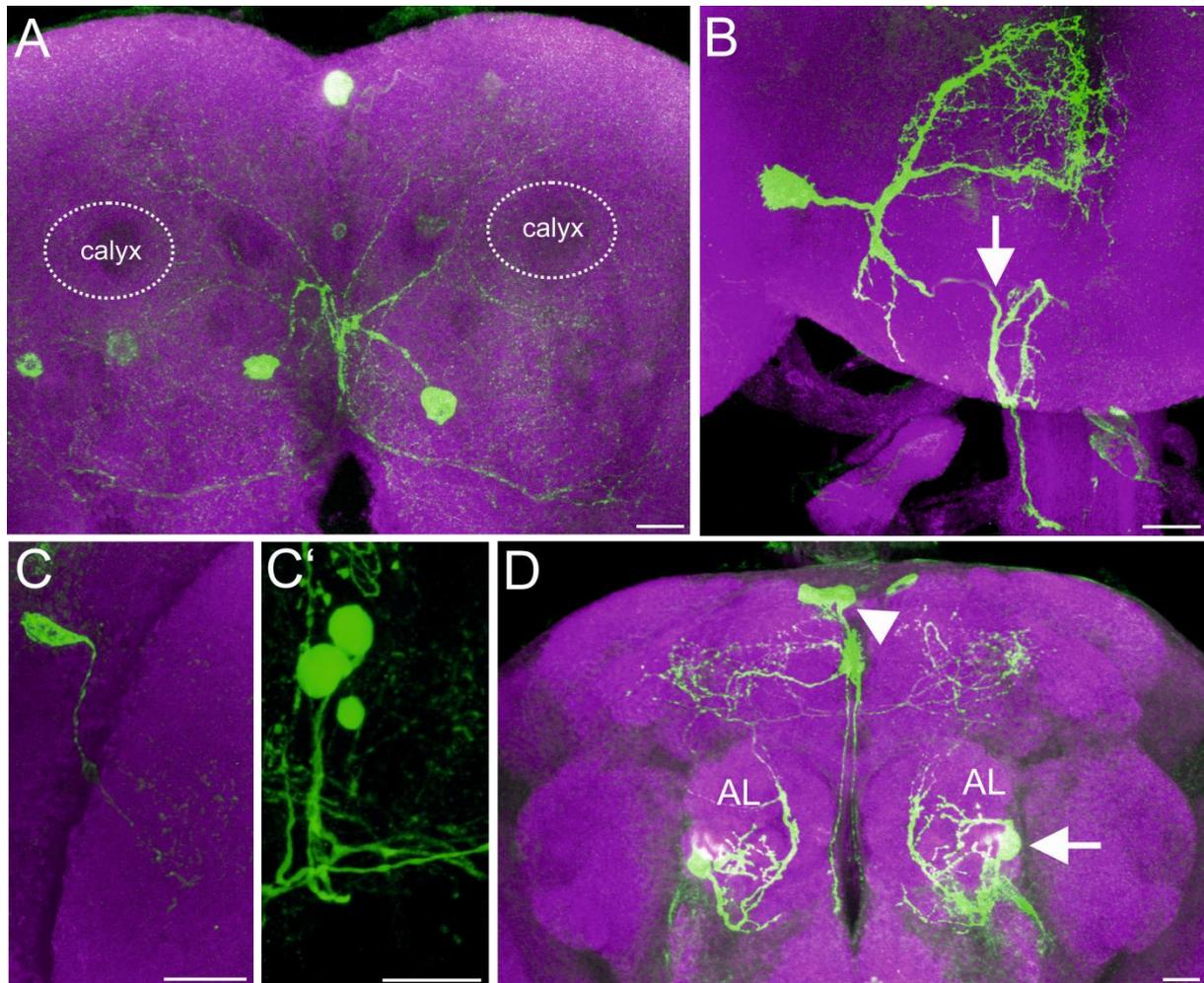

Figure 3.1.6.5 Neurons projection pattern of *SERT3*-GAL4/UAS-mCD8-GFP. (A) IP neuron projection pattern. One big neuron located on each side to IP cluster, it sent its axon to the middle line of the brain then three branches formed. One of the branch stretched following inner antennocerebral tract to lateral horn, another one reach to calyx then surround it by small fibers, the third one firstly follow middle line then turn to lobula. (B) SE1 projection pattern. One asteroid cell on the surface of SOG stretch its axon to the SOG then split one branch to the upper middle part of SOG and another one to thoracic ganglia. (C-C') One LP1 soma appeared at the posterior of the brain, it then project through the brain and merged to ventrolateral protocerebrum (C). The same pattern was observed in LP1 from *TPH*-GAL4 (C'). (D) Non-serotonergic neurons projection pattern. A cluster of neurons stayed on top of the brain middle line and sent axons until inside border of AL (arrow head). On surface of AL few cells stick together (arrow). They sent one branch to AL and another one crossed AL until superior lateral protocerebrum. (magenta=nc82, green=GFP, scale bar 20 μm)

Three clusters with six serotonergic neurons lead to decreased ethanol preference after disturbed dSERT function. Are these neurons specific for ethanol odour response or can they also change ethanol sensitivity or tolerance? To address this question *SERT3*-GAL4 was crossed to UAS-*SERTDN*-GFP then tested for ethanol sensitivity and tolerence. *SERT3*-GAL4/UAS-*SERTDN*-GFP displayed 21.8±2.9 min of MET1 which is similar level as *SERT3*-GAL4/+ (21.0±1.4) and UAS-*SERTDN*-GFP/+ (20.4±1.8) control flies (Fig.3.1.6.6A). The even MET1 suggests that prolonged serotonin signaling in *SERT3*-GAL4 dependent neurons could not change ethanol sensitivity. After second round of ethanol exposure *SERT3*-GAL4/UAS-*SERTDN*-GFP flies increased 28.5%±3.7% of MET which was not significantly different from two genetic control flies (Fig.3.1.6.6B). This result indicates that longer serotonin signaling in *SERT3*-dependent neurons could not alter ethanol tolerance and then further suggest the neurons in IP and LP1 cluster are specific for ethanol preference.
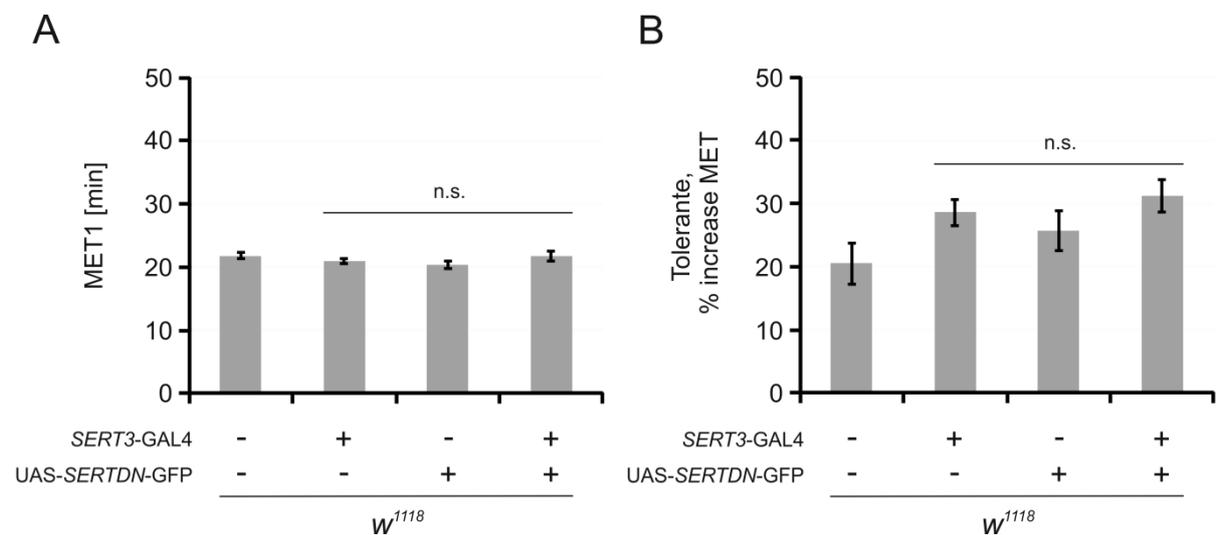

Figure 3.1.6.6 Disturbed SERT function in SERT3-GAL4 by expressing UAS-SERTDN-GFP did not change ethanol sensitivity and tolerance. (A) *SERT3*-GAL4/UAS-*SERTDN*-GFP (21.8±2.9) showed similar ethanol sensitivity as genetic control flies. (B) Increase of MET did not change significantly from *SERT3*-GAL4/UAS-*SERTDN*-GFP (28.5%±3.7%) to *SERT3*-GAL4/+ (27.1%±2.6%) and UAS-*SERTDN*-GFP/+ (25.7%±2.6%). (ANOVA posthoc test, n.s. $P > 0.05$, n=12).

3.1.7 Two opposite neuronal circuits modulates ethanol preference

In previous results one neuron in IP cluster and another one in LP1 cluster were proposed to be the serotonergic neurons that modulate ethanol preference. *TRH*-GAL4 was reported to drive 75-100% of serotonergic neurons in adult brain (Alekseyenko, et al., 2010). Therefore, when SERT function in *TRH*-GAL4 dependent neurons is disturbed, decreased ethanol

preference should be observed, if *TRH*-GAL4 contains the neurons from *SERT3*-GAL4. To test this idea, *TRH*-GAL4 flies were crossed to UAS-*SERTDN*-GFP then tested in two choice assay. Surprisingly *TRH*-GAL4/UAS-*SERTDN*-GAP flies displayed 0.32±0.05 of preference to ethanol which was not significantly different from two control groups (Fig. 3.1.7.1A, *P* >0.05). This result indicated that disturbed SERT function in *TRH*-GAL4 dependent neurons could not alter ethanol preference. There are three possible reasons to make this result. The first one is *TRH*-GAL4 only drives the non-function neurons than *SERT3*-GAL4; second is *TRH*-GAL4 drives different neurons which had opposite function; third one is *TRH*-GAL4 has two opposite neuronal circuits which compromised the effects. To identify the reason both *TRH*-GAL4 and *SERT3*-GAL4 constructs were introduced to the same genome then crossed with UAS-SERTDN-GFP. As showing in Fig. 3.1.7.1B *SERT3*-GAL4; *TRH*-GAL4/UAS-*SERTDN*-GFP displayed 0.60±0.09 of PI which was not significantly different from its two genetic controls. This data means another neuronal circuit exists independent of *SERT3*-GAL4 manner neurons.
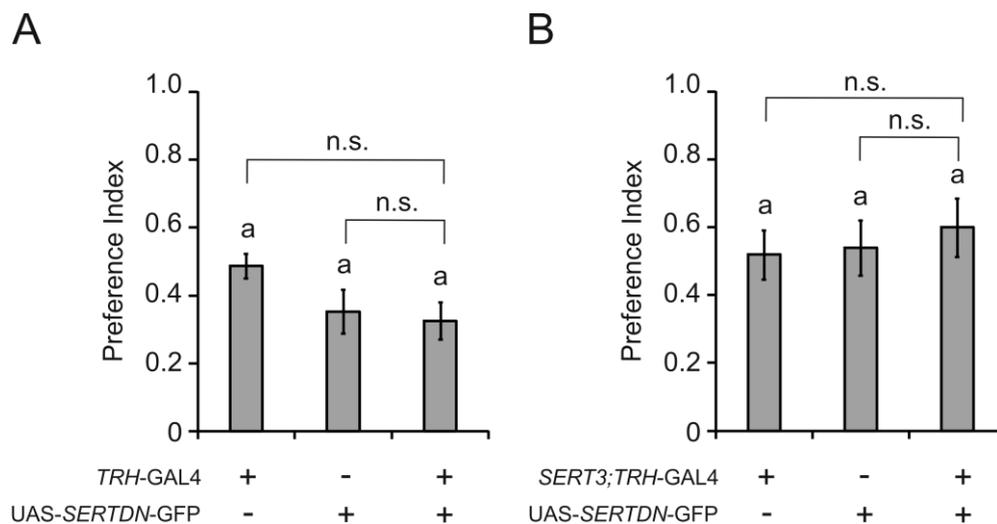

Figure 3.1.7.1 Additional neuronal circuit exists in *TRH*-GAL4 to balance ethanol preference effect. (A) *TRH*-GAL4/UAS-*SERTDN*-GFP had ethanol preference index of 0.32±0.05. That was not significantly different in contrast with two controls (*P* > 0.05, n=30-31) (B) *SERT3*-GAL4;*TRH*-GAL4/UAS-*SERTDN*-GFP displayed PI of 0.60±0.09 which is even to *SERT3*-GAL4;*TRH*-GAL4/+ and UAS-*SERTDN*-GFP/+ flies (*P* > 0.05, n=31-32). (ANOVA posthoc test, n.s. *P* > 0.05, nonparametric one sample sign test, a= different from random)

To verify whether *TRH*-GAL4 contained all the *SERT3*-GAL4 dependent neurons, *TRH*-GAL4 was crossed to UAS-mCD8-GFP. CNS of the adult flies was stained with GFP and 5-HT. 5-HT positive neurons in SE1, LP2, CSD and AP clusters were recognized, in anterior

part of the brain (Fig. 3.1.7.2A). In all these clusters found in 5-HT channel could be found in anterior brain of GFP channel (Fig. 3.1.7.2A'). The merge channel both 5-HT and GFP signal were well over lapping with each other (Fig. 3.1.7.2A'). In posterior part of the brain,
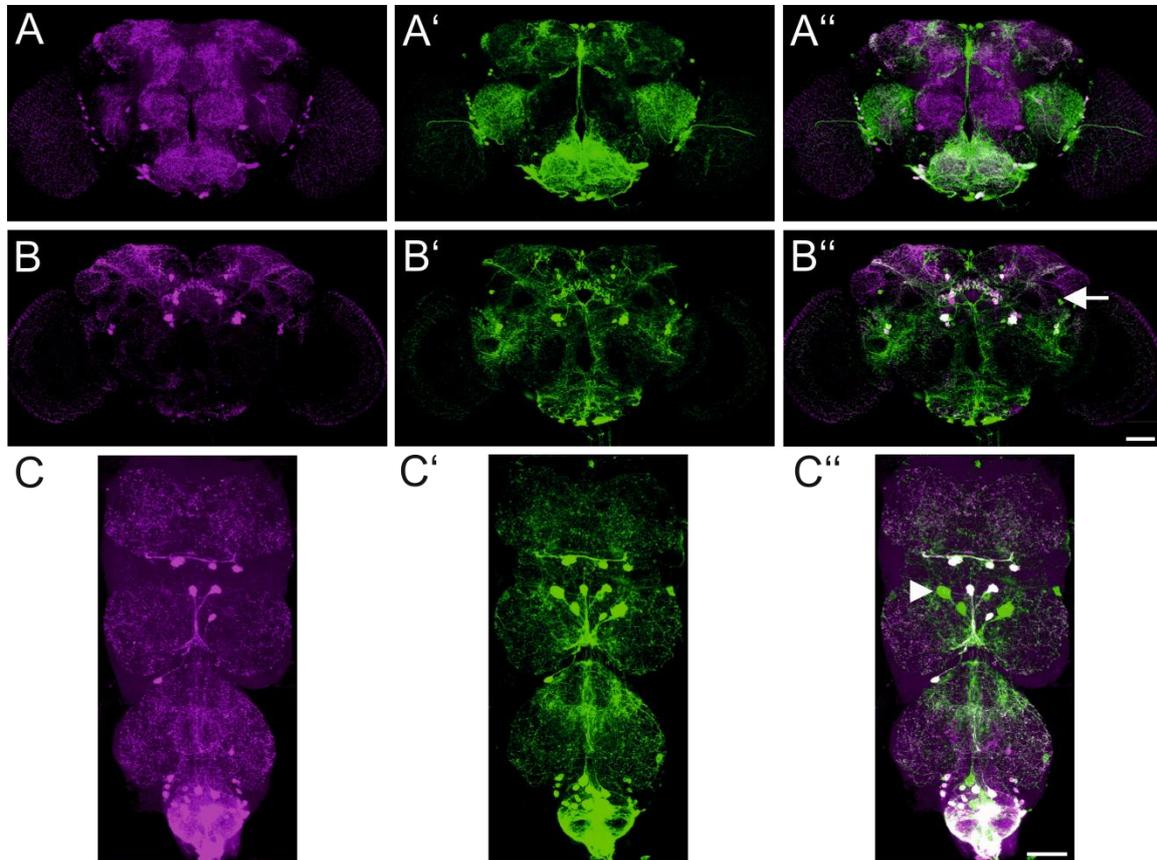

Figure 3.1.7.2 Co-localization of 5-HT and GFP in CNS of *TRH*-AGL4/UAS-mCD8-GFP flies. (A-A'') 5-HT and GFP colocalization in anterior part of brain. 5-HT positive neurons in SE1, LP2, CSD and AP clusters; GFP positive neurons co-labeling with most of 5-HT clusters. (B-B'') 5-HT and GFP co-localization in posterior of adult brain. SP1, SP2, IP and LP1 clusters and fanshape body co-express 5-HT and GFP. (C-C'') Overview of 5-HT and GFP expression pattern in adult thoracic ganglia. GFP signal could be detected through every segment in thoracic ganglia and abdominal ganglia. Most of the GFP cells were also 5-HT positive except some in Meso segment (arrow head). (magenta=5-HT, green = GFP, Scale bar 50μm)

fan-shaped body could clearly be seen in the upper middle part, 5-HT positive cells were found in SP1, SP2, IP and LP1 clusters (Fig. 3.1.7.2B); these clusters also show GFP positive neurons (Fig. 3.1.7.2B'). In the merge channel of posterior brain, all the serotonergic clusters were co-labeled with GFP positive cells, but there are also some only GFP positive neurons (Fig. 3.1.7.2B'' arrow). In thoracic ganglia, 5-HT positive cells are present in every segment clusters (Fig. 3.1.7.2C). GFP positive neurons could also be detected in Pro, Meso, Meta of

thoracic ganglia and abdominal ganglia (Fig. 3.1.7.2C'). In the merge channel, serotonergic cell and GFP cells were co-labeling with each other in different segment, but some neurons in Meso were only GFP positive (Fig. 3.1.7.2C'' arrow head).

Due to immuno-staining procedure and age of the flies, cell numbers for detection were variable. Therefore, numbers of different clusters from CNS were calculated in average to get an accurate expression pattern of *TRH*-GAL4. There are in total 44.0±0.3 of 5-HT positive cells in brain and 53.2±0.8 of GFP positive neurons in the brain. All 12 serotonergic clusters were found to be GFP positive (Fig. 3.1.7.3A). The non-serotonergic GFP positive neurons driven by *TRH*-GAL4 were not shown in the drawing. Expression pattern of *TRH*-GAL4 brain was summarized in the table of Figure 3.1.4.4A. Single cell clusters DP and CSD were

A

TRH-GAL4	5-HT ± STDEV	GFP ± STDEV	Merge ± STDEV	n
SP1	3.0 ± 0.0	3.5 ± 0.7	3.0 ± 0.2	24
SP2	5.2 ± 0.6	4.7 ± 0.9	3.6 ± 0.6	22
IP	6.9 ± 0.7	7.1 ± 1.0	5.2 ± 1.0	19
LP1	2.0 ± 0.0	9.6 ± 1.7	2.0 ± 0.0	22
LP2	10.1 ± 1.4	12.5 ± 1.7	7.1 ± 1.5	25
LP3	2.7 ± 0.6	1.0 ± 1.2	0.3 ± 0.5	16
SE1	4.1 ± 0.3	4.1 ± 0.4	4.1 ± 0.3	28
SE2	2.0 ± 0.2	2.0 ± 0.5	1.9 ± 0.5	22
SE3	3.0 ± 0.0	3.0 ± 0.0	3.0 ± 0.0	23
AP	2.9 ± 0.3	3.7 ± 1.3	2.1 ± 0.5	26
DP	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	15
CSD	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	21

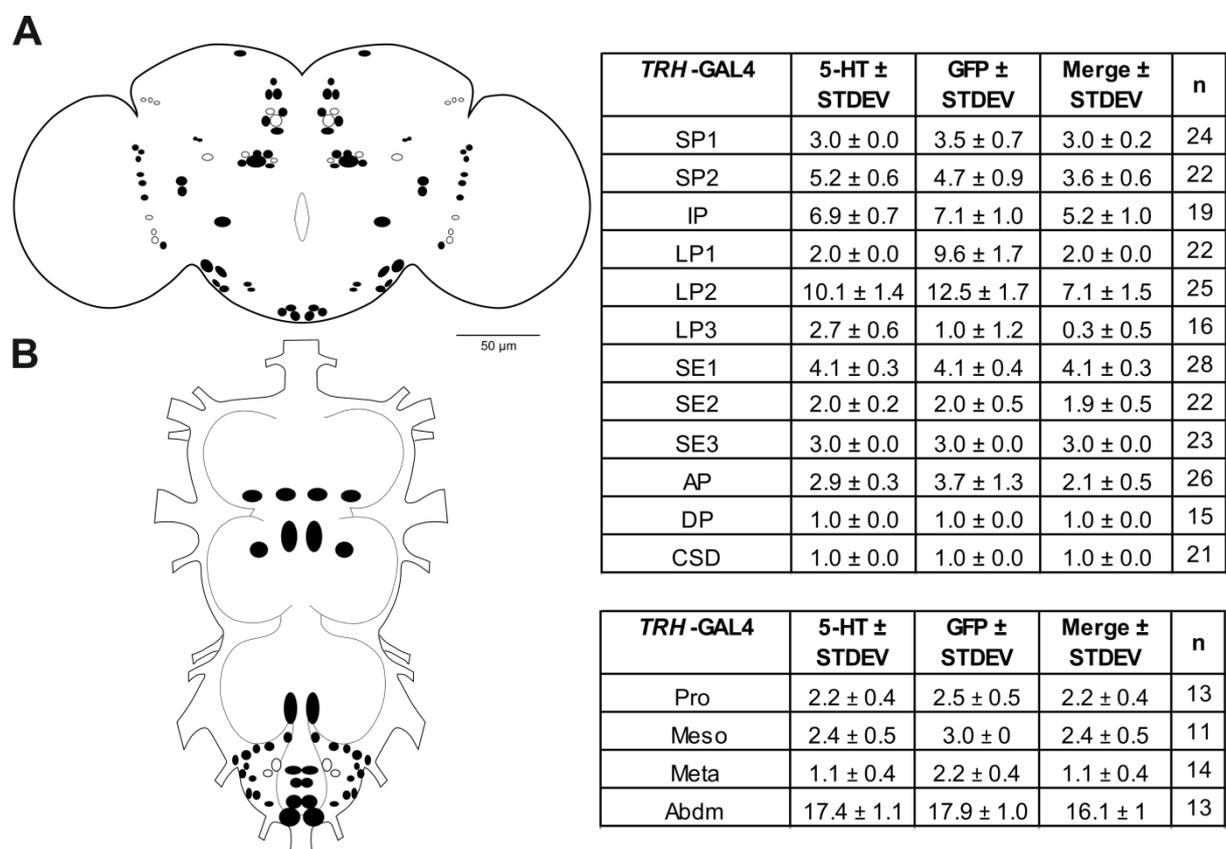| *TRH*-GAL4	5-HT ± STDEV	GFP ± STDEV	Merge ± STDEV	n
Pro	2.2 ± 0.4	2.5 ± 0.5	2.2 ± 0.4	13
Meso	2.4 ± 0.5	3.0 ± 0	2.4 ± 0.5	11
Meta	1.1 ± 0.4	2.2 ± 0.4	1.1 ± 0.4	14
Abdm	17.4 ± 1.1	17.9 ± 1.0	16.1 ± 1	13

Figure 3.1.7.3 Schematic diagram and neuron analysis of *TRH*-GAL4 CNS. (A) There are 78% of serotonergic neurons co-labeling with GFP in the brain. All serotonergic clusters have GFP positive neurons. 5-HT positive neurons in DP, LP1, CSD, and SE1-3 clusters are all over lapping with GFP (n=15-28). (B) GFP positive cells present in all the serotonergic clusters in thoracic ganglia. 100% of GFP and 5-HT co-labeling was seen in Pro, Meso, Meta. In Abdm two serotonin positive cells on each side did not express GFP (n=11-14). (circles= 5-HT positive, black dots=overlap of GFP and 5-HT)

found in both 5-HT and GFP channels. In the lateral protocerebrum cluster LP1 all the 5-HT positive cells (2 cells) are co-labeling with GFP. In the sub-esophagus region, four 5-HT positive cells from SE1 cluster were found to be GFP positive as well. The same is true for SE2 (2 cells) and SE3 (3cells) cluster. There are three 5-HT cells found in the anterior protocerebrum (AP cluster), only two small neurons being colocalized with GFP. 70% of LP2 cluster express both GFP and 5-HT. In superior protocerebrum, the entire three 5-HT positive cells in the SP1 cluster are GFP positive. In SP2, three out of five 5-HT positive neurons had GFP expression. In the IP cluster one round big cell and four small surrounding cells are both 5-HT and GFP positive.

Expression pattern of 5-HT and GFP cells in thoracic ganglia is showing in Figure 3.1.7.3B. In Prothoracic neuromere (Pro) four GFP cells lined at the end of the segment, they are all serotonergic. In Meso cluster four GFP neurons out of six are overlap with 5-HT cells. In Meta segment, two pairs of GFP positive cells were found, but only one of them was serotonergic. Most of the 5-HT positive neurons in abdominal segment were also GFP positive (92%); only two pairs of serotonergic cells did not have GFP expression. This data revealed *TRH*-GAL4 can drive UAS construct expression in 83% of serotonergic neurons from all the clusters in adult CNS.

Even though *TRH*-GAL4 can cover most of the serotonergic neurons, the accurate morphology of *SERT3*-GAL4 containing clusters in it were not clear. To verify whether *SERT3*-GAL4 was including in *TRH*-GAL4, *TRH*-GAL4 was crossed to UAS-mCD8-GFP or brought to brainbow system. Firstly, *TRH*-GAL4/UAS-mCD8-GFP flies with GFP and 5-HT staining was closely analyzed. Two 5-HT positive LP1 cells were co-labelled with GFP signal (Fig.3.1.7.4A''). In SE1 cluster, there were four 5-HT positive neurons. All that four cell were overlapping with GFP signal; the big asteroid neuron was included (Fig.3.1.7.4B' arrow). Most 5-HT positive neurons overlap with GFP in IP cluster. One big GFP cell from center was serotonergic (Fig.3.1.7.4C'' arrow head). The higher magnification analysis of LP1, SE1 and IP clusters in *TRH*-GAL4 showed that except two small cells in IP it can drive all the cells in these clusters. To better visualize the single cell projection pattern in *TRH*-GAL4. Cre; *TRH*-GAL4 flies were generated and then crossed with brainbow flies. In Projection pattern of SE1 cell, the big asteroid cell (arrow) in SE1 cluster sent two branches to upper middle part of SOG and another one to the lower part of it (Fig.3.1.7.4D). In projection pattern of IP cells,

big neuron form IP cluster (arrow) extend its axon to the middle line then separate in to three branches: one branch stretch down to the lobula, another one goes up following inner anternnocerebral track and middle one go to calyx(Fig.3.1.7.4E). When this result is compared with Fig.3.1.6.5, it is obvious to see that *TRH*-Gal4 contains all the serotonergic neurons that can be driven by *SERT3*-GAL4.
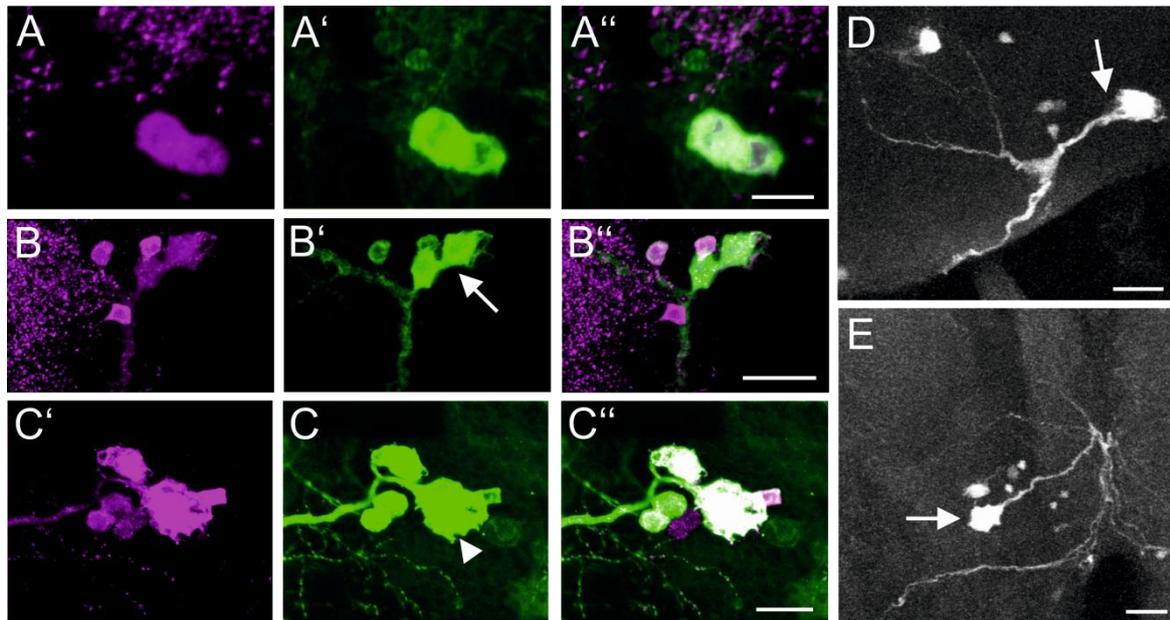

Figure 3.1.7.4 Close morphology of LP1, SE1 and IP cluster in *TRH*-GAL4 dependent neurons. (A-A") Two 5-HT positive LP1 cells were co-labeling with GFP signal. (B-B") Four 5-HT positive neurons in SE1 cluster overlapping with GFP positive cells. The big asteroid neuron (arrow) in SE1 expresses both GFP and 5-HT. (C-C") Most 5-HT signal overlaps with GFP in IP cluster. One big GFP cell from center was serotonergic (arrow head). (D) Projection pattern of SE1 cell. The big asteroid cell (arrow) in SE1 cluster sent two branches to upper middle part of SOG and another one to the low part of it. (E) Projection pattern of IP cells. One big neuron form IP cluster (arrow) extends its axon to the middle line then separate in to three branches. One branch stretches down to the lobula, the middle one goes to calyx. (magenta=5-HT, green=GFP, scale bar 20 μm)

Two opposing neuronal circuits were found in *TRH*-GAL4 that modulates ethanol preference. *RN2*-GAL4 had been shown to drive one pair of CSD neuron and send branch in antennal lobe and calyx (Roy et al., 2007). To find out whether CSD neurons is the possible neuronal circuit that contradict with IP and LP1 neurons in modulating ethanol preference, *RN2*-GAL4;*SERT3*-GAL4 flies were crossed to UAS-*SERTDN*-GFP and then tested for preference. *RN2*-GAL4; *SERT3*-GAL4/UAS-*SERTDN*-GFP flies showed 0.57±0.12 of preference to 5% ethanol which was similar compared to two control groups (Fig. 3.1.7.4A, *P* >0.05).
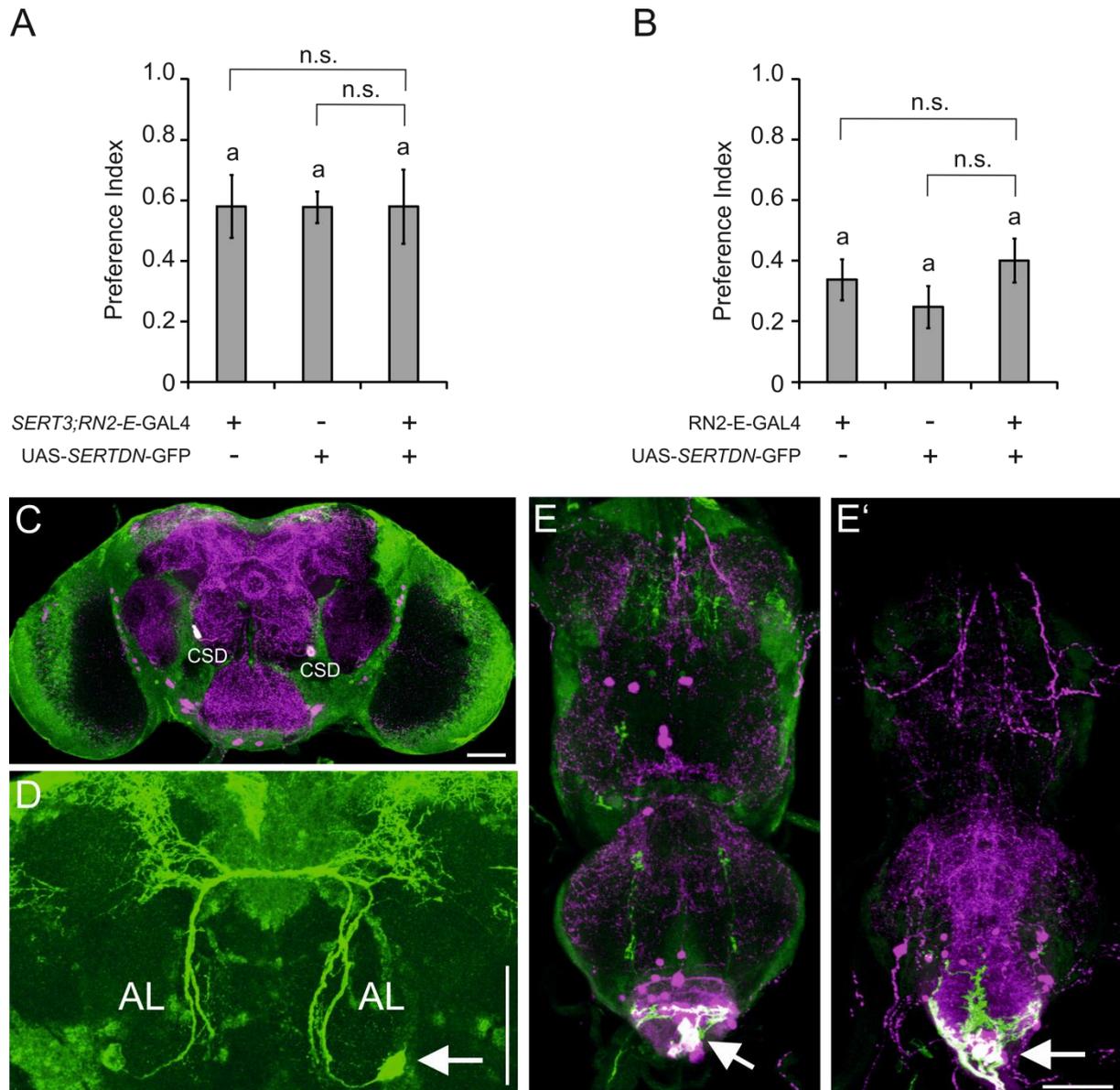
Figure 3.1.7.5 CSD neurons and a cluster of cells in abdominal ganglia compromise ethanol preference change. (A) *SERT3*-GAL4; *RN2-E*-GAL4/UAS-*SERTDN*-GFP had ethanol preference index of 0.57±0.12. That was not significantly different compared with two controls ($P > 0.05$, n=30-31) (B) *RN2-E*-GAL4/UAS-*SERTDN*-GFP displayed PI of 0.4±0.07 which is higher, but not significantly different to *RN2-E*-GAL4/+ (0.33±0.07) and UAS-*SERTDN*-GFP/+ (0.24±0.07) flies ($P> 0.05$, n=45-58). (C) Co-labeling of GFP and 5-HT in *RN2-E*-GAL4/UAS-mCD8-GFP. Only one pair of CSD neurons were found in GFP channel which overlaps with 5-HT signal. (D) Projection pattern of CSD neuron. CSD neuron project to the higher center then branches to calyx and protocerebrum. (E-E') Co-labeling of GFP and 5-HT in thoracic ganglia and abdominal ganglia. GFP and 5-HT positive neurons overlap in anterior of abdominal ganglia (E, arrow). At posterior of abdominal ganglia, both GFP and 5-HT signal was detected in the same cells and fibers (E', arrow). (magenta=5-HT, green=GFP, ANOVA posthoc test, n.s. $P > 0.05$, nonparametric one sample sign test, a= different from random, scale bar 50 μm).

This result indicates that disturbed SERT function in *RN2*-GAL4; *SERT3*-GAL4 dependent neurons could not alter ethanol preference, suggesting that additional neurons from *RN2*-GAL4 compromised the effect from *SERT3*-GAL4 dependent neurons. Serotonin has a modulation role in olfactory processing in antennal lobe (Dacks et al., 2009). CSD neuron had been showed to be serotonergic in moth (Hill et al., 2002). To test whether disturbed SERT function in CSD neuron could induce ethanol preference change, *RN2*-GAL4 flies were crossed to UAS-*SERTDN*-GFP and tested in two choice assay. *RN2*-GAL4/UAS-*SERTDN*-GFP exhibit 0.4 ± 0.07 of PI to ethanol (Fig. 3.1.7.5B) which was a slight increase of PI compared to *RN2*-GAL4/+ (0.33 ± 0.07) and UAS-*SERTDN*-GFP/+ (0.24 ± 0.07), but the difference was not significantly different from each other ($P > 0.05$). This data suggests that disturbed SERT function in CSD neuron could not significantly influence ethanol preference.

To further confirm that CSD neuron in *Drosophila* is serotonergic, *RN2-E*-GAL4 was crossed to UAS-mCD8-GFP and stained with GFP and 5-HT. There is only one CSD neuron on each side of antennal lobe, both 5-HT and GFP signal could be detected in CSD (Fig. 3.1.7.5C). CSD neuron projects its axon to the higher centre then branch to calyx and lateral protocerebrum on each side of the brain; finally it will stop at the contra lateral antennal lobe (Fig. 3.1.7.5D). In the lower part of abdominal ganglia, a cluster of GFP positive neurons co-labelling with 5-HT signal at the middle line is seen (Fig. 3.1.7.5E arrow). These clusters arborize one branch up along the middle line and stop in the border of Meso; another branch follow the rim goes up and down of the rim (Fig. 3.1.7.5E' arrow). This data revealed that *RN2-E*-GAL4 could not only drive the expression of CSD neurons but the neurons in abdominal ganglia and both CSD neurons and the neuron in abdominal ganglia are serotonergic.

Both *TRH*-GAL4 and *RN2*-E-GAL4 could neutralize ethanol preference change caused by insufficient SERT function in *SERT3*-GAL4 dependent neurons. To identify the accurate neuron that compromise preference change, serotonergic neurons that could be driven by different GAL4 driver lines were compared.
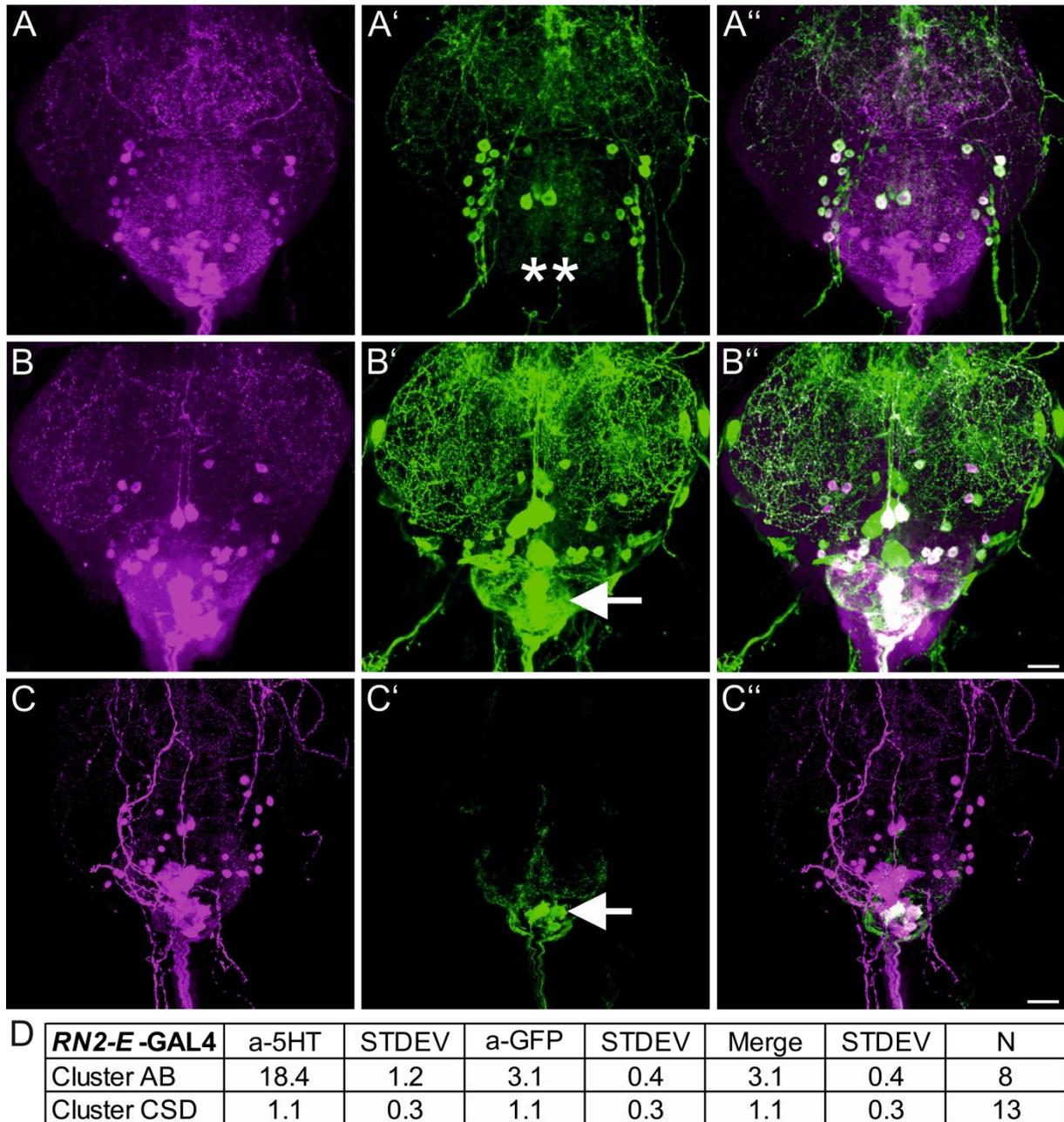
RN2-E-GAL4	a-5HT	STDEV	a-GFP	STDEV	Merge	STDEV	N
Cluster AB	18.4	1.2	3.1	0.4	3.1	0.4	8
Cluster CSD	1.1	0.3	1.1	0.3	1.1	0.3	13

Figure 3.1.7.6 Comparison of serotonergic neuron in abdominal ganglia of TPH, TRH and RN2-E dependent neurons. (A-A'') In low middle part of *TPH*-GAL4/UAS-mCD8-GFP no GFP positive neuron could be detected (asterik). (B-B') In abdominal ganglia of *TPH*-GAL4/UAS-mCD8-GFP, clusters of GFP positive cells (B' arrow) were observed and they all overlap with 5-HT (B''). (C-C'') A cluster of GFP positive cells in the end of Abdm of *RN2-E-GAL4*/UAS-mCD8-GFP and they were all co-labeling with 5-HT (C''). (D) Summary of *RN2-E-*GAL4 dependent serotonergic neurons. (magenta=5-HT, green=GFP, scale bar 20 μm)

In the brain of *RN2-E*-GAL4 there is only one CSD neuron which is also included in *TRH*-GAL4. Furthermore, both *SERT3*-GAL4 and *TPH*-GAL4 did not contain CSD neuron. That suggests CSD neuron is required for modulating preference. *SERT3*-GAL4 did not drive any

serotonergic neuron expression in thoracic ganglia. *TPH*-GAL4 which have similar ethanol preference change like *SERT3*-GAL4 after SERT disruption could not drive any cell expression in lower part of abdominal ganglia (Fig. 3.1.7.6 asterisk). This result leaves neurons in Abdm to be the candidate. There are six GFP positive neurons (Fig. 3.1.7.6D) at the bottom of *RN2-E*-GAL4/UAS-mCD8-GFP (Fig. 3.1.7.6C) which are also found in *TRH*-GAL4 (Fig. 3.1.7.6B'). This data suggests that CSD neuron in the brain combined with six neurons at the end of abdominal ganglia modulates the ethanol preference effect from IP and LP1 cluster.

3.1.8 Disruption of SERT function in DPM neurons or activation of SERT3 dependent neurons does not alter ethanol preference

The CSD neurons project to mushroom bodies and antennal lobes (Roy et al., 2007). To determine whether CSD neurons are directly connected to olfactory receptor neurons (ORNs) a broadly expressed co-receptor driver Or83b-LexA (Lai and Lee, 2006) was used together with RN2-P-GAL4 to drive *Drosophila* GFP reconstitution across synaptic partner (GRASP) expression (Gordon and Scott, 2009). In the brain there was no GFP signal that could be detected (Fig. 3.1.8A). In glomerulus of antennal lobes where ORNs and projection neurons form synapses there was also no GFP signal (Fig. 3.1.8A'). This indicates CSD neurons are not directly connected to ORNs. To test whether DPM neurons which can innervate to mushroom body (Lee et al., 2011) are involved in ethanol preference, *C316*-GAL4 was crossed to UAS-*SERTDN*-GFP. Flies showed 0.28±0.08 of PI which was not significantly different from control (Fig. 3.1.8B $P > 0.05$), suggest that DPM neurons are not required for ethanol preference. These results provide a strong hint that olfactory pathway does not induce ethanol odor evoked decision making. To determine whether neuronal activity in *SERT3*-GAL4 dependent neurons is sufficient to induce preference, light frequency of 40 Hz followed by 8 Hz (Schneider et al., 2012) was used to activate UAS-*ChR2* (Channelrhodopsin-2) under SERT3-GAL4 driver. Blue light could activate neurons when CHR2 combining with all-trans retinal (Schroll et al., 2006). When offering flies with the same food odour during light activation experimental flies fed with retinal did not show any preference (-0.12±0.1) as the controls (Fig. 3.1.8C). This data suggests that activating neurons in SERT3-GAL4 dependent neurons is not sufficient to induce preference.
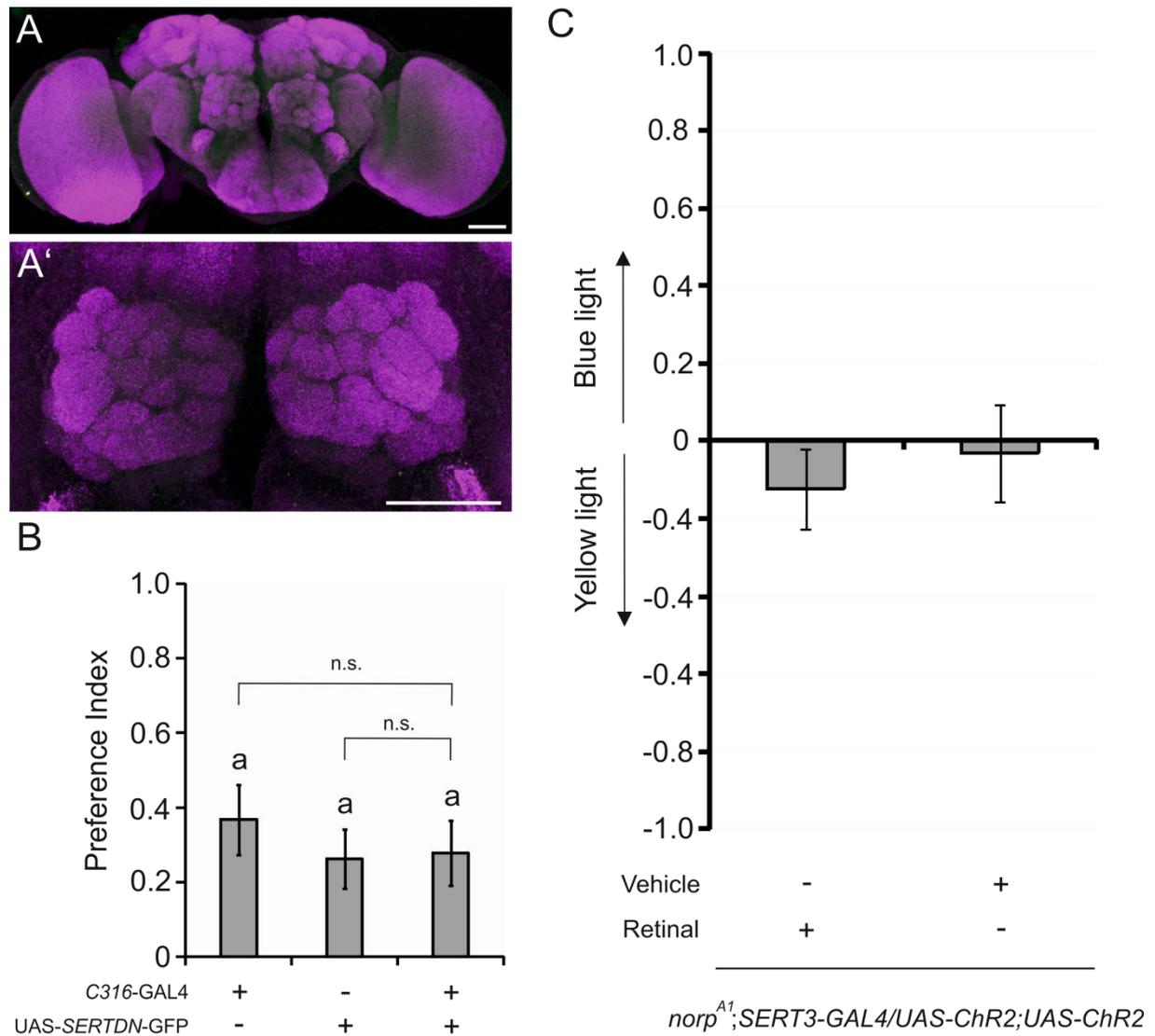
Figure 3.1.8 Disturbing SERT function in DPM neurons or neuronal activation of SERT3 dependent neuron does not alter ethanol preference. (A-A') CSD neuron dose not connect to olfactory receptor neurons. No GFP signal could be detected in *RN2-P*-GAL4 /LexAop-GFP[11]; *Or83b*-LexA/ UAS-GFP[1-10] brain and in antennal lobe (imaged for GFP fluorescence, magenta=nc82, scale bar 50 μm). (B) No ethanol preference change was obtained by disturbing SERT function in DPM neurons (modified after Goldman, 2012; ANOVA posthoc test, n.s. $P > 0.05$; nonparametric one sample sign test, a= different from random). (C) Neuronal activation of SERT3 dependent neuron does not alter ethanol preference (PI of norp[A1], *SERT3*-GAL4/UAS-*ChR2*; UAS-*ChR2* with retinal -0.12±0.1 and with vehicle -0.02±0.12 $P > 0.05$; n=25-26).

3.2 dSERT mutants are impaired in negative geotaxis

In ethanol induced preference assay dSERT16 mutant could not enter the trap which makes it impossible to analyse the preference change caused by dSERT mutants. It is not clear whether dSERT16 is a mutant in other behavioral paradigm. It has been showed that dVMAT mutants

have mild defect in anti-geotaxis behavior (Simon et al., 2009). However, there is no direct evidence to show the relationship between serotonin and negative gravitaxis.

3.2.1 dSERT mutants are impaired in negative geotaxis

In ethanol induced preference assay dSERT16 mutant could not enter the trap which make it impossible to analysis the preference change caused by dSERT mutants. It is not clear whether dSERT16 is a mutant in other behavioral paradigm. There is no direct evidence to show the relationship between serotonin and negative gravitaxis. To answer the question whether serotonin is involved in gravitaxis behavior and what role it played, *dSERT* mutants were tested. In this assay, flies are firstly transferred into the first tube then shake them down 5 times. After each shake the upper part of the apparatus is moved one tube back, the flies can choose to climb up then moved to the next or stay. Before *dSERT* mutants, *dSERT1* and w^{1118} was tested to confirm if they have the same phenotype in negative geotaxis. Both wild type flies and dSERT1 tend to accumulate in the last 2 tubes of the assay gadget (>70% Fig. 3.2.1A). Less than 7% of those two genotypes left in the first group and about 20% of flies stay in the second group (Fig. 3.2.1A). From all the three groups there are no significant difference found between w^{1118} and *dSERT1* (*P* >0.05). *dSERT1* was generated using the same procedure as the other mutants. Since *dSERT*1 was generated in the same mutagenesis, did not show molecular genetic changes and behave similar to W^{1118} flies, it was further used as a control. There is no significant difference between *dSERT1* and *dSERT10* in each group (*P* >0.05, Fig. 3.2.1B). In contrast, *dSERT16* and *dSERT18* show severe defects in negative geotaxis behavior (Fig. 3.2.1C, D). Almost half of the *dSERT16* mutant flies (43%) cannot move to the second group in comparison to control only 2% of flies that stay in the first group (Fig. 3.2.1 C). It is a significant defect in climbing ability compare to *dSERT1* (*P* <0.001). Even though there are only 24% of *dSERT16* stayed in the middle group, that still showed a decrease in anti-geotaxis ability (*P* <0.05, Fig. 3.2.1C). Contrast from *dSERT1* in which 80% can climb to the last group, only 32% of *dSERT16* moved to the last group (Fig. 3.2.1 C). In dSERT18, 65% of flies move to the third group, 28% in the middle group and only 6% in the first group. *dSERT18* showed the trend to move to the end, but compared to *dSERT1* control every group is significantly different (Fig. 3.2.1 C).
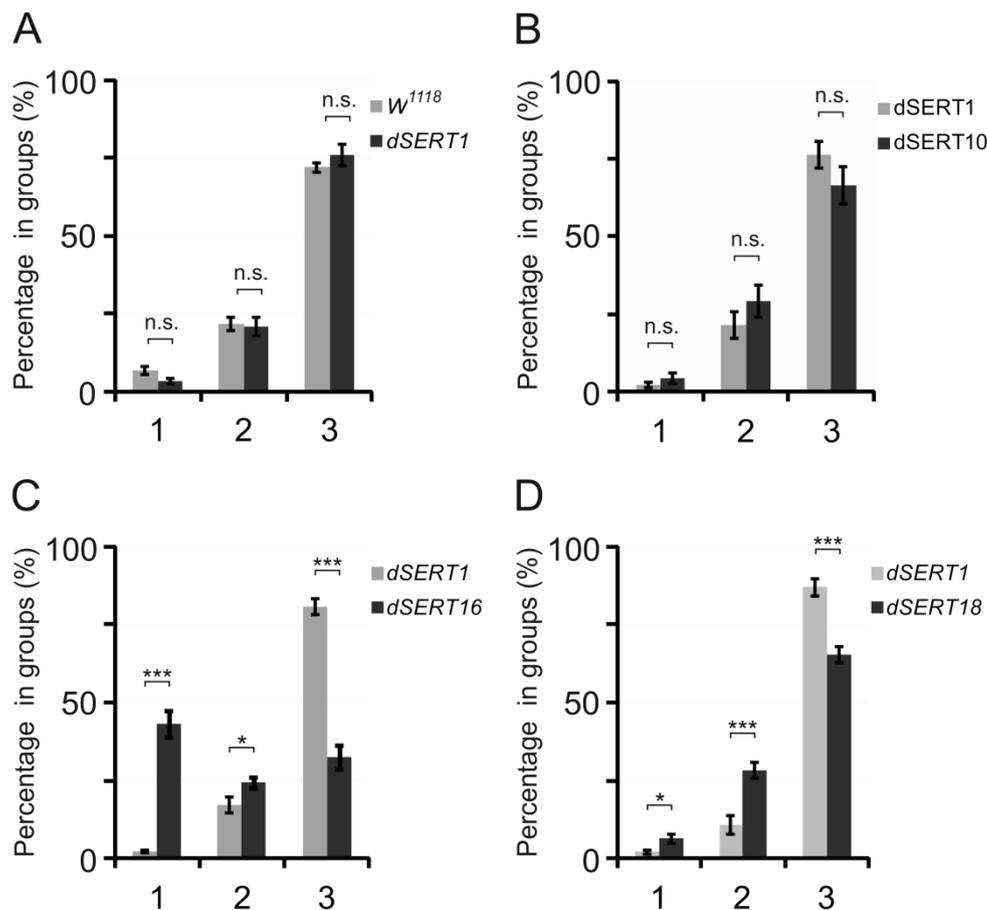
Fig 3.2.1 Deplete dSERT caused defects in negative geotaxis. (A) *dSERT1* and W^{1118} show same phenotype in climbing ability. In each group, there is no significant difference between W^{1118} and *dSERT1* (B) *dSERT10* did not shows significant defect in anti-geotaxis. (C) *dSERT16* has severe problem with moving against gravity. Only 32% of the flies can climb to the last group. Half of the flies stay in the first group. Percentage of flies in each group is significantly different from control ($P < 0.05$). (D) *dSERT18* has mild defect in gravitaxis. *dSERT18* has more flies stay in the first two groups. Compare to the control, flies in the last group are significantly less. (Student T-test * $P < 0.05$, ** $P < 0.01$, ***$p < 0.001$; n = 7-9)

In the first group *dSERT16* mutants have bigger distribution ratio but smaller ratio in the last group ($P < 0.001$) that is similar to *dSERT18*. There is no significant difference between *dSERT18* and *dSERT16* in the middle group. All of the dSERT gravitaxis behaviours demonstrate that flies with loss of dSERT protein leads to a defect in anti-geotaxis ability. Whether that is due to prolonged signalling of serotonin in the synaptic cleft or not still need to be further tested. *dSERT16* has the most severe problem in gravitaxis. Among different dSERT mutants the more severe the *dSERT* loss is, the worse the climbing ability it gets. Suggesting that *dSERT16* is null allele *dSERT10* and *dSERT18* are hypomorphs.

3.2.2 Disturbed SERT function in limited neurons did not affect negative geotaxis behaviour

Previous data showed that dSERT mutants have defects in a negative geotaxis assay. dSERT mutants disable dSERT function in all the serotonergic neurons, it is not clear which neurons are crucial for negative geotaxis. To further understand how serotonin transporter regulates this behaviour and in which neurons dSERT exert modulation, different tools are used to increase or decrease serotonin level in different serotonergic cells. *TPH*-GAL4 covers 54% serotonergic neurons in the brain and 75% in the thoracic ganglia which showed in the previous data (Fig. 3.1.4.4). The colocalization of 5-HT and GFP positive neurons in *TPH*-GAL4/UAS-mCD8-GFP flies do not match completely. One possibility to explain this is that part of the GFP positive neurons could be dopaminergic, since TPH is also required in dopamine synthesis (Coleman and Neckameyer, 2005). To test whether dopaminergic neurons include in *TPH*-GAL4, TH and GFP were stained in *TPH*-GAL4/UAS-*mCD8*-GFP brains. Dopaminergic neurons present in anterior and posterior of the adult brain, clusters surround SOG are beside serotonin clusters. Between optic lobe and protocerebrum there are also TH positive cells, but there is no colocalization between GFP and TH (Fig. 3.2.2.1 A-A''). TH positive cells can be detected in Pro, Meso and Meta segment of thoracic ganglia. Moreover, dopaminergic neurons in thoracic ganglia located in similar positions as serotonergic neurons (Fig. 3.2.2.1 B-B''). Since there is no detectable overlap between TH and GFP, it is clearly indicates that the *TPH*-GAL4 line do not drive the expression of dopaminergic neurons.
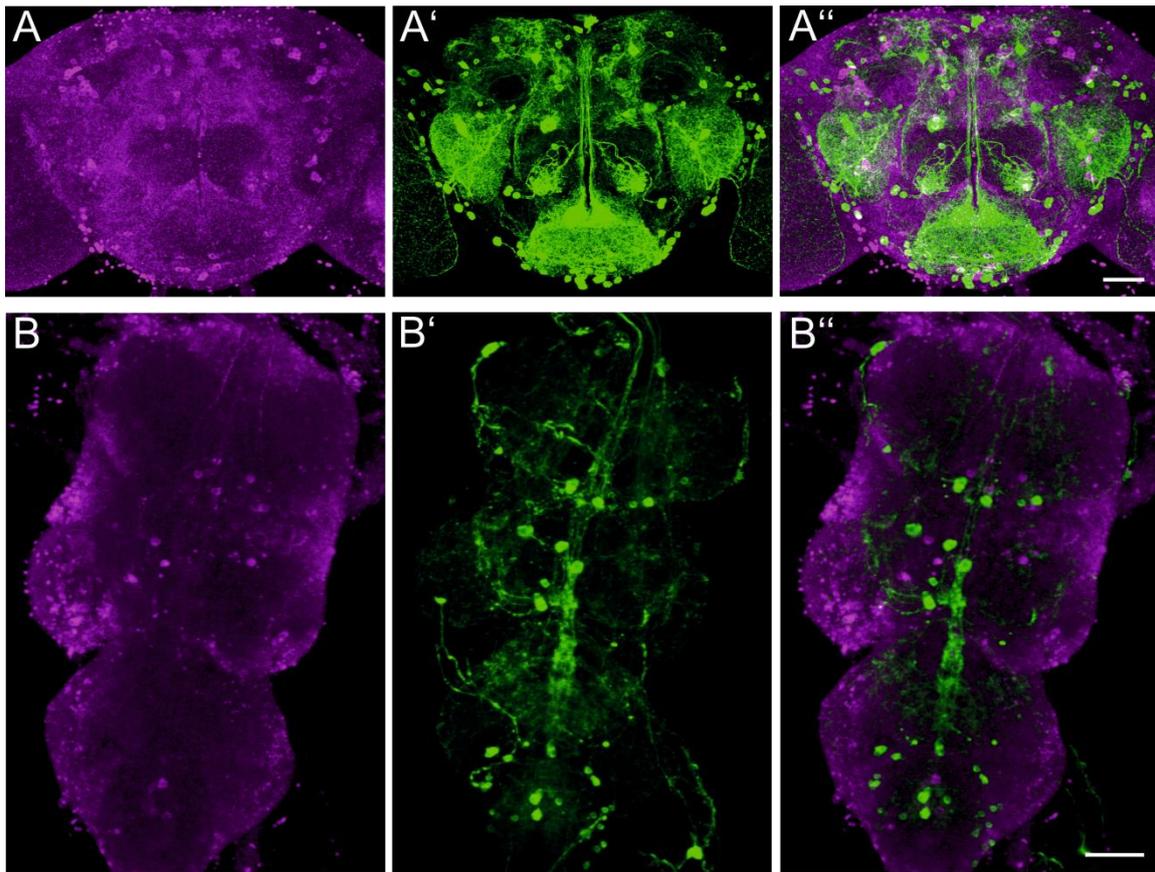
Figure 3.2.2.1 No colocalization of TH and GFP in *TPH*-GAL4*/*UAS-*mCD8*-GFP CNS. (A) Staining of GFP and TH in *TPH*-GAL4*/*UAS-*mCD8*-GFP brain. GFP staining (Aʻ) cover different serotonergic clusters are not colocalized (Aʻʻ) with TH (A) positive neurons. (B) GFP and TH are not overlapped with each other in thoracic ganglia. Dopaminergic neurons exist in different segment of thoracic ganglia (B), but not overlap with serotonergic neurons (Bʻʻ). (A, B is TH staining; Aʻ, Bʻ is GFP staining; Aʻʻ, Bʻʻ is merge of GFP and TH signal. scale bar is 50μm).

Aiming at unravelling the behaviour of flies over expressing dSERT in TPH neurons, UAS-*SERT*-GFP flies were crossed with *TPH*-GAL4 flies. Ectopic expression of dSERT could lead to increased 5-HT reuptake (Park et al., 2006). So a reduced serotonin signalling is expected for over expression of dSERT in TPH driven neurons. The over expression flies showed robust ability to move until the last group. Rarely flies were found in the first group, only 0.9 % of flies stay in the middle group (Fig. 3.2.2.2 A). There is no significant difference found with the genetic controls. This result indicates that decreased serotonin level in the synapse cliff of TPH driven neurons did not affect geotaxis. Chang et al. (2006) over expressed UAS-*DVMAT* in *Ddc*-GAL4 diver line and a defect in negative geotaxis was observed. They also showed that over expression of DVMAT increase serotonin storage. Thus, increased serotonin storage could be the key factor that suppresses negative gravitaxis ability.
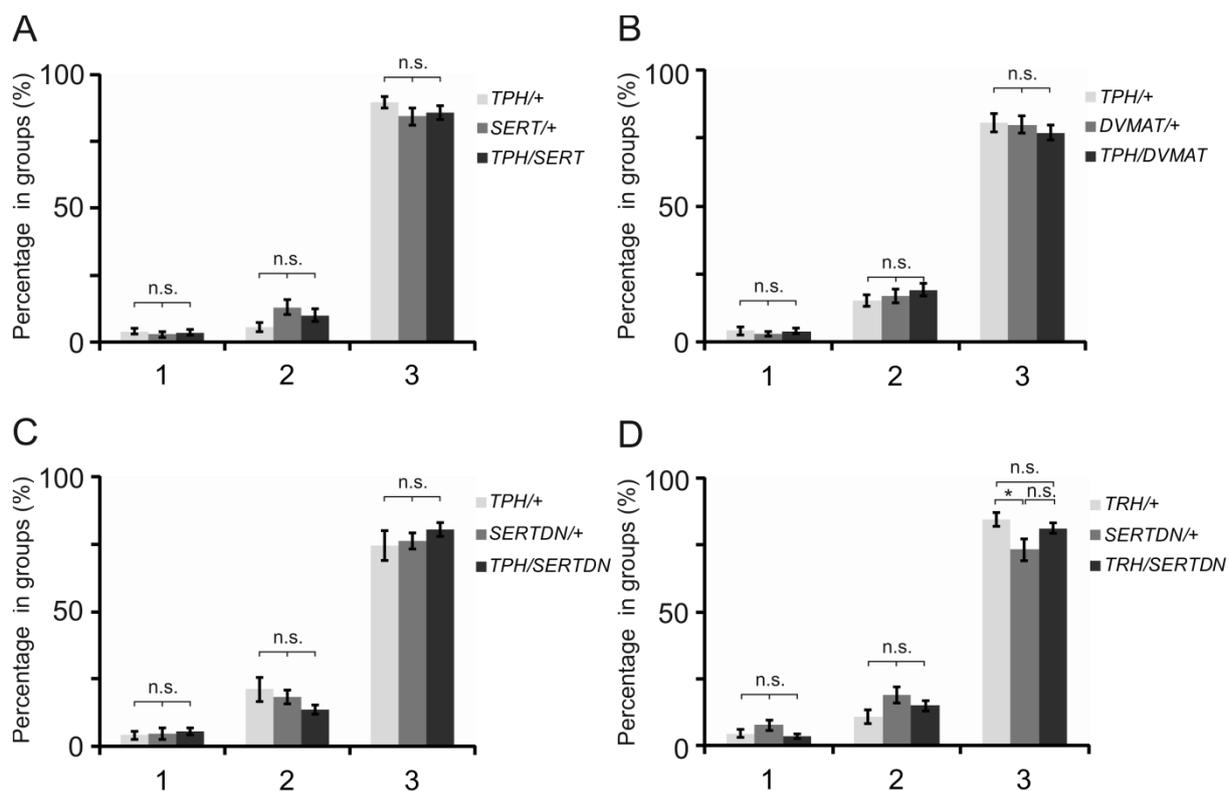
Figure 3.2.2.2 Increase or decrease of 5-HT reuptake in broad serotonin neurons had no significant effect on negative geotaxis behaviour. (A) Over expression of SERT in TPH dependent neurons caused no change in climbing behaviour. (B) Increased serotonin storage by expressing DVMAT in TPH neurons cannot change the behaviour of climbing against the gravity. (C) Disrupting serotonin reuptake in TPH dependent neurons by SERTDN expression caused no significant difference in climbing. (D) Expressing SERTDN in TRH dependent neurons cannot change negative geotaxis. More flies of TPH control than SERTDN control in the last group. (ANOVA posthoc test n.s. $P > 0.05$, * $P \leq 0.05$, n = 7-11).

Besides serotonergic neurons, *Ddc*-GAL4 also drives DVMAT over expression in dopaminergic neurons. To specify over expression pattern of DVMAT in serotonegic neurons, UAS-*DVMAT* flies were crossed with *TPH*-GAL4 flies. After increased serotonin storage, flies exhibit normal negative geotaxis compared to genetic controls: most (77%) of them climbing to the last group, 19% lagged in the middle and only 4% stay in the first group(Fig. 3.2.2.2 B). More serotonin storage may not cause a direct excess of 5-HT release. This could be the reason why no behaviour change in DVMAT over expression flies is seen. One direct way to increase serotonin effects is to elongate the duration of serotonin in the synaptic cleft. In the aim of suppressing dSERT reuptake function, UAS-*SERTDN*-GFP construct was generated (Ritze, 2007). In this construct two putative serotonin binding sites were mutated. Expression of SERTDN in serotonergic neurons caused reduction of 5-HT level in the cytoplasm (Kiaser, 2009). To disable serotonin transporter in serotonergic neurons, UAS-

SERTDN-GFP was crossed to *TPH*-GAL4. The results show that most of the experimental flies could move to the last group of tubes leaving almost no flies behind in the first group. This also holds true checked against the genetic controls (Fig. 3.2.2.2 C). When expressing UAS-*SERTDN* construct in serotonergic neurons, serotonin reuptake in these cells are greatly suppressed (Kaiser, 2009). If the set of neurons are directly responsible for geotaxis, similar phenotype as dSERT mutants should be observed. Defect in climbing ability did not record in *TPH*-GAL4/UAS-*SERTDN*-GFP flies. This strongly address that *TPH*-GAL4 does not drive the right set of neurons which control geotaxis. In the sense of getting a broader expression of SERTDN in serotonergic neurons, *TRH*-GAL4 was chosen. *TRH*-GAL4 has been shown 75%-100% of serotonin clusters co-labelling and no overlap with dopaminergic neurons (Alekseyenko et al., 2010). Most of the flies climb to the last group when SERTDN express in TRH dependent neurons, left 15% in the middle and 3% in the first group (Fig. 3.2.2.2D). Similar phenotype exists in the genetic controls. In the last group there is no significant difference between SERTDN/TRH and controls, but TRH control had more flies than SERTDN control (Fig. 3.2.2.2D, *P* >0.05). This result implies low 5-HT up take in TRH neurons did not change negative geotaxis behaviour.

In all tested subgroups alerting SERT function did not interfere with negative geotaxis suggesting that the phenotype observed with the Ddc-GAL4; UAS-*DVMAT* night not modulated by the same set of neurons in TPH or TRH. Since Ddc-GAL4 only drive serotonergic neuron expressing in LP2 and SP2 cells (Chang et al., 2006), a specific driver line which can drive less serotonergic neurons expression is needed.

3.2.3 A subset of serotonergic neurons is involved in negative geotaxis

Altering serotonin levels in relatively broad set of serotonergic neurons cannot alter negative geotaxis. Among dSERT mutants different phenotypes were also clearly exhibited. Above two contradictory results provide a clue that there are two components in negative geotaxis modulation. Chang et al. (2006) suggested that serotonergic neurons innervated to central complex might modulate locomotion behaviour. Therefore a small amount of serotonergic cells could be directly linked to geotaxis response. In our lab a serotonergic driver line *SERT3*-GAL4 was generated (Herb, 2005). In this driver line only 3-5 serotonergic neurons per brain hemisphere and there is no 5-HT positive neuron found in the thoracic ganglia. It was also been shown that CSD neuron response to mechanical stimulus in moth (Dacks et al., 2008). To address whether these neurons play role in climbing or negative geotaxis, *SERT3*-

GAL4; UAS-*SERTDN*-GFP were tested for negative geotaxis assay. There are slight differences between *SERT3*-GAL4/UAS-*SERTDN*-GFP and two controls in the second and third groups, but not statistically significant (Fig. 3.2.3.1 A). To better understand the function of CSD neuron in gravitaxis, RN2-GAL4 (Fujioka et al., 2003) was crossed to UAS-*SERTDN*-GFP. RN2-GAL4/ UAS-*SERTDN* flies had no change in negative geotaxis climbing phenotype than controls (Fig. 3.2.3.1 B). Those data above suggest that reduction of SERT function in SERT3 or RN2-GAL4 dependent neurons does not interfere with negative geotaxis.
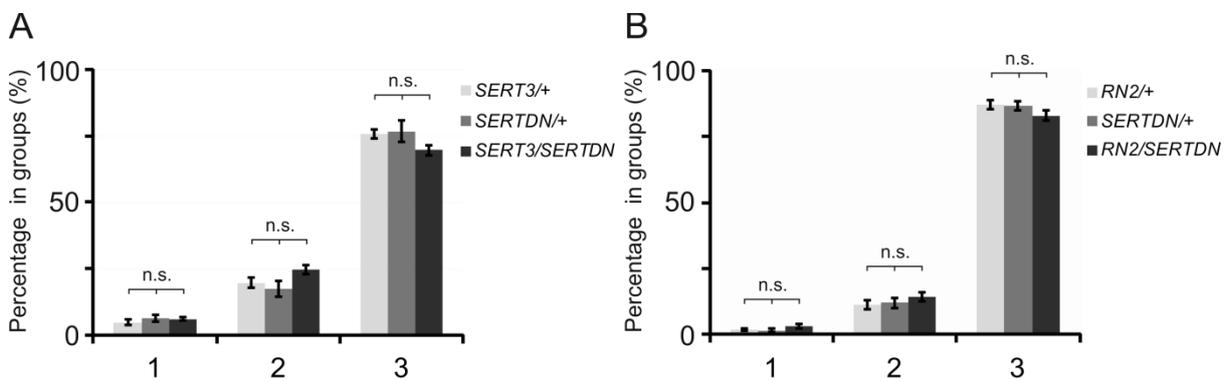
Figure 3.2.3.1 Disruption of serotonin reuptake in small set of serotonergic cells does not change negative gravitaxis. (A) Reduction of serotoin in SERT3 depended neurons do not affect negative gravitaxis. (B) CSD neuron is not sufficient for controlling geotaxis. Flies show intact geotaxis climbing ability after disturb SERT function in RN2-E depended neurons. (ANOVA posthoc test n.s. *P* >0.05; n = 8-11)
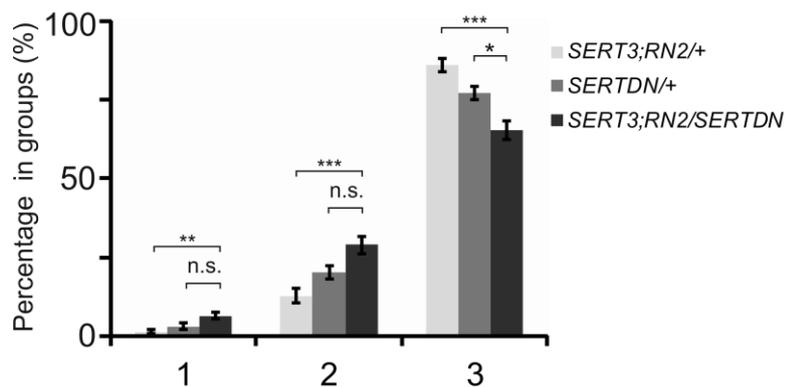

Figure 3.2.3.2 Disturbing 5-HT reuptake in both brain and abdominal ganglia decreased anti-geotaxis climging. Less *SERT3;RN2/SERTDN* flies can climb till the last group (65%, *P* <0.05). In the first and second group *SERT3;RN2/SERTDN* (29%) are significnatly different from *SERT3;RN2/+* (*P* <0.05). (ANOVA posthoc test, n.s. *P* > 0.05,* *P* < 0.05, ** *P* < 0.01, ***P* < 0.001; n = 13).

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Disturbed SERT function in SERT3 or RN2 dependent neurons could not alter negative geotaxis. One possibility is that the neurons in this two drives are not enough to make a response decision. Based on this idea, flies containing *SERT3*-GAL4 were brought to *RN2*-GAL4 back ground. After crossing this line to UAS-*SERTDN*-GFP, decreased climbing ability against geotaxis was observed. Only 65% of the total *SERT3*-GAL4; *RN2*-GAL4/UAS-SERTDN flies can climb to the last group which is significant decrease compare to controls (Fig. 3.2.3.2, *P* <0.05). Nearly 30% of flies stay in the middle group and left the rest in the first group. These data indicate that depleting serotonin reuptake in SERT3 and RN2-E dependent neurons disrupt negative geotaxis.

4. Discussion

4.1 dSERT mutants show normal olfactory ethanol preference

In serotonin signaling SERT plays a critical role since it can mover the released serotonin and transport it back for recycling (Murphy et al., 2004). It has been reported that there are around 80 serotonergic neurons in each adult brain (Giang et al., 2011). Therefore, modify SERT function will lead to directly change in serotonin signaling. Park et al. (2006) reported that ectopic expression of UAS-dSERT construct could up take 5-HT from the extracellular region. However, the specific genetic tool to research on lost of dSERT function is missing. To understand the role of serotonin in ethanol evoked preference, *dSERT* mutants were generated (Kaiser, 2009). The levels of *dSERT* transcript expression in the mutants are varied (Ruppert, 2008), and observed different dSERT protein level. No dSERT protein could be detected when use RIPA buffer to extract the total protein from 15 flies head. This might be due to the fact that only a limited number of serotonergic neurons expressing the dSERT transporter are found in the head of the flies. After increasing the amount of protein using 1000 fly head and in particular isolating only the membrane fraction, the expected expression domain of the the dSERT protein, a expected 65kDa protein could be detected (see 3.1.1). This is consistent with the identified rSERT protein from the hippocampus of the brain (Huff et al., 2013). SERT belongs to SLC6 family and SLC6 gene family is defined to have 12 trans-membrane domains (Thimgan et al., 2006). Consistent with the fact that dSERT protein is a membrane integrated protein, dSERT was only detected in membrane but not cytoplasm fraction of the protein isolation.

When *dSERT* mutants with different size of genomic lesions are tested for ethanol odor preference, dSERT10 and dSERT18 with lack of SERT protein level and unaltered neighboring gene exhibited preference to ethanol containing food odors to the same extend as control flies. The majority of *dSERT16* did not even enter the traps. The dSERT16 mutants that do enter the traps show same degree of ethanol preference as controls (see 3.1.2). SERT knock out mouse also showed same level of ethanol preference as wild type (Boyce-Rustay, 2006). The dSERT16 mutants carry the largest deletion suggesting that dSERT16 is the strongest allele of dSERT. At least three possibilities exist in why dSERT16 do not enter the trap by showing response to ethonal. First one is that they cannot smell the odors. Second dSERT16 are unable to decide between two similar complex odor sources. Third they cannot

convert the decision into locomotor. Since when offer dSERT16 with simple odor choices water and food odor they showed preference to food (Schläger, 2013), indicating dSERT16 could distinguish simple odor. After increase the complexity of the odor choices, even only offer dSERT16 with food and 5% of ethanol they could not decide. Failed to go in to the trap might due to the motivation change under complex odor. Even thought dSERT16 could make the decision under two simple orders, only half of the tested group flies could totally showed preference and trapped. This result suggests that execution of motor behavior also involved in odor preference. One possible explanation is that the *dSERT16* gene deletion is the largest, so after translation there are less dSERT protein could insert onto the membrane, even the same amount of dSERT protein on *dSERT16* membrane the functionality of them also lower than other mutants. In addition, other behavioral defects are also observed which is associated with the functional loss of dSERT. Negative geotaxis test for dSERT16 demonstrate that dSERT16 have severe defect in climbing, similarly dSERT18 flies' climbing ability also impaired (see 3.2.1), but the observed results suggest that dSERT16 is the stronger allele than dSERT18.

When dSERT mutants are tested for their ethanol sensitivity and tolerance, *dSERT16* are more resistant to ethanol but show normal tolerance. The *dSERT10* are more sensitive and more tolerant, dSERT18 get similar sensitivity and tolerance as control (Kaiser, 2009). The ethanol sensitive and resistant data of dSERT mutants suggests the different deletion could cause different SERT activity. In rodents, SERT knockout and heterozygote mutant were more sensitive to serotonin uptake inhibitor than wild type (Montañez, et al., 2003). Study on SERT knockout mice displayed increased extracellular level of 5-HT; the increase of serotonin level further proved to be gene does dependent (Mathews et al., 2004). This is also supported by knockout mice exhibited increased sensitivity to ethanol induce sedation (Boyce-Rustay, 2006). SERT knockout mice decreased in ethanol consumption compare to heterozygote and wild type (Lamb and Daws, 2013). Interestingly the original P-element insertion line do4388 showed no preference for ethanol containing food odors suggesting that a potential dSERT mutant exist. Ethanol tolerance phenotype was also altered in do4388 which indicated the P-element insertion might influence the expression and function of the neighboring gene.

4.2 Serotonin acts as a negative regulator in olfactory ethanol preference

Increase serotonin level in the flies' head by feeding 45mM of 5-HTP, as a result flies lost the preference to 5% of ethanol (see 3.1.4.1). When fed flies only with 5mM of 5-HTP, flies had slightly decreased preference but not significantly different from control. This result suggests increased serotonin level will suppress ethanol preference. This result is similar to the finding in mice which block serotonin reuptake by fluoxetine reduce alcohol intake (Kelai, 2003). However, it is not clear in which level serotonin suppresses ethanol preference, since both endogenous release of 5HT in serotonergic neurons or excess serotonin in the synaptic cleft could prolong serotonin signaling.

Expression of a SERT with mutated 5-HT binding sites under the control of an UAS sequence in a TPH-GAL4 dependent manner results in a reduction of 5-HT in serotonergic neurons (Kaiser, 2009). This reduction of reuptake causes decreased ethanol preference (see 3.1.4.2). This reduction might due to conformation change of dSERT, since research in rat showed that SERT internal domains conformation change is important for serotonin transport (Androutsellis-Theotokis and Rudnich, 2002). These are two evidence supporting that access serotonin in the synaptic cleft regulates ethanol preference. Secondly over expression of DVMAT could slightly decrease ethanol preference but not significantly different from the controls. Chang et al. (2006) showed that 5-HT level was up regulated by 20% in the brain when over express DVMAT in both serotonergic and dopaminergic neurons. DVMAT is not only for 5-HT transportation but also other neuronal amine so the specificity of serotonin to DVMAT is lower than serotonin to its transporter. Furthermore Increased serotonin storage might not necessarily increase 5HT in the synaptic cleft. In the DVMAT over expression pattern, the dSERT function was not altered; hence serotonin reuptake could compromise the increase of serotonin level. Which suggest dSERT function is required in ethanol preference and further indicates increased serotonin level in synaptic cleft rather than cytoplasm induced preference change. SERT knockout mice show lower preference to ethanol than control further supports this result (Kelai, 2003). This result is also consisting with the observation that over expression of normal dSERT protein in a TPH dependent manner does not alter ethanol preference (3.1.4.2). However, changes of internal 5-HT by over expression of DVMAT does not alter behavior either. Over expressing of DVMAT in TPH dependent

neurons could not alter climbing ability of fly. When over expression of DVMAT in TPH manner neurons flies show similar ethanol sensitivity and ethanol tolerance as controls (see 3.1.4.5). In conclusion serotonin plays a role of negative modulator in olfactory preference-high serotonin level suppresses ethanol preference and it exerts its function as increases or prolongs synaptic signaling.

4.3 dSERT and serotonin expression are variable in adult CNS

In larvae and adult brains, it has been shown dSERT only express in serotonergic neurons (Giang et al., 2011). During *Drosophila* development from third larvae to adult serotonergic neuron will increase from 85 to 106 (Vallés and White, 1988). Whether the dSERT positive neurons change after the cells increase is unknown. In adult CNS, dSERT staining could be seen in most of the serotonergic neurons in the brain. A close look at dSERT and serotonin co-labeling pattern in SE2 cells demonstrate that serotonin spread all over the cells, dSERT mainly exist on the membrane since the cells were hollow. Some brightly stained dots on the cells surface were observed in both dSERT and 5-HT staining and they next to each other. In the synaptic boutons dSERT and 5-HT staining are adjacent with each other. At the end of abdominal ganglia dSERT staining could not be observed (see 3.1.5.4). Besides the absent of dSERT positive neurons at end of abdominal ganglia, LP3 cluster which could be seen in 5HT staining becomes always hard to see in dSERT staining. SP3 cluster was first reported by Giang et al. (2011); it could not be detected in every stained adult brain. In larvae stage this cluster was neither been recorded in 5-HT staining nor dSERT (Huser et al., 2012; Giang et al., 2011). It has been reported both in mammals and *Drosophila* that DAT could transport elevated external serotonin (Zhou et al., 2002; Daubert et al., 2010). In rat brain, serotonin needs to distribute to the release site and defuse to the target to affect circuit (Bunin and Wightman, 1998). Therefore, the positive signal detected in SE3 cluster could be the staining of serotonin diffused in dopaminergic cells. This idea could also apply to why there is only serotonin staining but no dSERT signal at the bottom of abdominal ganglia.

Besides the expected serotonergic neurons some extra dots and round circles was also observed on the CNS surface of dSERT staining. Glia cell was detected in lamina cell body layer when use riboprobe from SLC6 family to hybridize with *Drosophila* CNS (Thimgan, et al., 2006). The SLC6 family conserved in amino acid residues and they also have similar structures. The dSERT staining from surface of CNS have high possibility to be glia cells. In

dSERT and 5-HT co-labeling pattern two dSERT positive cells but not serotonin positive was found in LP2 cluster (see 3.1.5.4). Week staining of 5-HT could not explain this phenomenon, since other LP2 cells were well stained. Yuan et al. (2005) showed clock cells (ventral lateral cells) are close to serotonergic cells which named as LP2. In the same time they also showed flies in dark condition got significant decreased of serotonin level in the head. In rats different serotonin level was reported based on circadian rhythm (Jakota and Kalyani, 2008). Light exposure might influence serotonin expression then further represent the cell number variation after staining. It is highly possible that the two extra dSERT positive cells are serotonergic cells which can response to light and dark cycle. The absent of 5-HT staining might due to deplete of serotonin after the dark stage.

4.4 Two serotonergic clusters determined ethanol preference

For serotonin positive cells analysis in *Drosophila* CNS varies numbers were reported, thus the clusters location is relatively stable (Vallés and White, 1988; Sitaraman et al., 2008; Alekseyenko et al., 2010; Giang et al., 2011; Huser et al., 2012). For example, there are two cells found in SE2 cluster but others reported three (Giang et al., 2011) and one cell (Alekseyenko et al. 2010) in the same cluster. Some other clusters like SP1, SP2 and IP cluster was firstly describe by Vallés and White (1988) were named PMP in research of Alekseyenko et al.(2010) and Sitaraman et al. (2008). To minimize the influence from cell variation average number from different brain clusters were used. The analysis of the GAL4 expression domain of the *TPH*-GAL4 driver line suggest that odor evoked ethanol preference uncovered a set of 41 serotonergic neurons in adult CNS. That covered 14 out of 16 serotongeric clusters (see 3.1.4.4). Within these set of neurons the candidate neurons are contained that might mediated olfactory ethanol preference. Increase extracellular serotonin level by over express the UAS-SERTDN in *SERT3*-GAL4 construct lead to reduced ethanol preference (Gräber, 2012). The newly generated *SERT3*-GAL4 line utilizes a promoter fragment of the *SERT* gene to direct GAL4 expression. Based on the SERT exclusively expressed in serotonergic neurons (see 3.1.5.4), the serotonergic neurons found dependent on *SERT3*-GAL4 are those putative neurons responsible for ethanol preference change. The phenotypic analysis of the GAL4 expression domain of the *SERT3*-GAL4 line revealed that in three serotonergic and four non serotongeric clusters GAL4 is expressed. Despite the fact that non serotongeric neurons are found in the thorax, no serotongeric neuron could be

manipulated in thoracic ganglia using the *SERT3*-GAL4 line. Absent of serotonergic neurons in thoracic ganglia have a big advantage to specifically understand the neurons function in the brain since the influence of the thorax and abdomen could be excluded. One seronergic cell found in SE1 cluster of *SERT3*-GAL4 did not showed in *TPH*-GAL4 which is not surprising since the *SERT* gene is under the control of different transcription factors than the *Tph* Gene. That indicates SE1 is not required for ethanol preference. These SE1 cells sent one branch to the SOG another one descending to the thoracic ganglia. In the similar region a pair of motor neurons which identified to response for proboscis extension also arborized in to SOG (Gordon and Scott, 2009). SE1 most likely works as a motor neuron than response to ethanol odor. In LP1 cluster, there is one serotonergic neuron in SERT3 manner but two in TPH dependent neurons. Three *Ddc*-GAL4 positive neurons reported in LP1 cluster (PLP) and all were overlapping with serotonin (Sitaraman et al., 2008). Difference in number of serotonergic cells in same cluster among different GAL4 driver lines suggest the potential influence from the construct insertion. LP1 neuron projects through the cleavage between central brain and optic lobe brain and end at ventrolateral protocerebrum. The serotonergic LP1 localized in similar position as *Drosophila* pigment dispersing factor neurons which is require for circadian rhythms (Renn et al., 1999). Quan et al. (2005) also reported these clock cells are close to serotonergic neurons which suggesting LP1 cluster might involved in circadian rhythms. In IP cluster a neuron with a large soma project one of the branches to lateral horn, another one reaches to mushroom body calyx then surround it with small fibers, the third arborize to lobula. Mushroom body is the higher center for chemosensory response. It was found the inner antennocerebral projection neurons connect the antennal lobe with the lateral horn and the calyx of the mushroom body (Tanaka et al., 2004). It is possible that IP cluster modulate ethanol preference through mushroom body. Mushroom body is important for olfactory learning and memory, a pair of serotonergic neurons DPM neurons innervated to mushroom body could modulate olfactory associated memory (Lee et al., 2011). Projection neuron CSD also shows the arborization to calyx (Roy et al., 2007). A reasonable assumption for reduced ethanol preference would be serotonergic IP neurons interact with mushroom body suppress the decision. Ethanol preference, sensitivity and tolerance were related to alcohol dehydrogenase (Adh) the key enzyme for ethanol metabolizing (Ogueta, et al., 2010). Ethanol sensitivity and tolerance did not altered after disturb dSERT function in *SERT3*-GAL4 (see 3.1.6.6). That indicating altered SERT function did not affect ethanol metabolizing. Further suggest under the normal ethanol metabolizing ethanol preference and

tolerance undergo different modulating pathway. Therefore, the serotonergic neurons in *SERT3*-GAL4 were specific for modulating ethanol preference. In conclusion IP neuron and LP1 neuron are the putative neurons that controlling ethanol induced preference. Further experiments are required to distinguish the function between IP and LP1 neuron.

4.5 Ethanol preference is modulated by two opposing serotonergic neural circuits

In olfactory induced behavior, olfactory input is required (Schneider et al., 2012). Olfactory sensory neurons which expressing different receptor and the general expressed co-receptor (Orco) are primary for *Drosophila* odor perception (Larsson et al., 2004; Kaupp, 2010). The olfactory sensory neurons project their axons to glomerulus in antennal lobe (Vosshall et al., 2000). Projection neurons innervate in to different glomerulus form synapses in Kenyon cells in mushroom calyx or surpass it and end in lateral horn (Keen and Waddell, 2007). In *SERT3*-GAL4 IP neurons which arborized surrounding mushroom calyx were the putative neurons that modulate ethanol preference. However, there was not clear the serotonin influence in the olfactory input. When increase synaptic serotonin level by disturbing dSERT function in *TRH*-GAL4 drive line which covered 78% of serotonergic neurons in the brain, ethanol preference did not changed. The phenomenon could be easily understand if in *TRH*-GAL4 there was no IP and LP1 cluster which were suppressing ethanol preference. Surprisingly, *TRH*-GAL4 contains all the serotonergic neurons that could be driven by *SERT3*-GAL4 (see 3.1.7.4). To verify the preference change was not due to the strength of different GAL4 expression, *SERT3*-GAL4 was brought into *TRH*-GAL4 back ground. When over express UAS-*SERTDN* in *SERT3*-GAL4/*TRH*-GAL4, flies did not show different ethanol preference to controls. Addition serotonergic neuronal circuits exist in *TRH*-GAL4 to neutralize the effect from IP and LP1 would be a reasonable explanation. Comparison of *TPH*-GAL4 and *TRH*-GAL4 suggested CSD, abdominal ganglia and DP are potential clusters to counteract with IP and LP1 cluster. CSD neurons which find out to be serotonergic in moth (Dacks et al., 2006) innervate into glomeruli in antennal lobe and branched in mushroom body (Hill et al., 2002). In *Drosophila*, RN2-E-GAL4 which generated by using a promoter fragment from segmentation gene *even skipped* (Fujioka et al., 2003) could drive CSD neuron expression (Roy et al., 2007). If CSD neuron involved in ethanol preference modulation, flies will not show preference change when co-express *SERT3*-GAL4/RN2-E-GAL4 with UAS-*SERTDN*.

This expected result was observed which confirmed the existent of the opposing serotonergic neural circuits. However, when expressing UAS-*SERTDN* in RN2-E-GAL4 ethanol preference was not altered. In addition of neurons in the brain, RN2-E-GAL4 also drives a cluster of serotonergic neurons in abdominal ganglia. That clusters of neurons projecting downward which could be the same serotonergic neurons that project to male reproductive apparatus (Lee et al., 2001). In dSERT staining there was also no dSERT signal in these clusters. In this regard, the serotonergic neurons in the end abdominal ganglia should not involve in ethanol induced preference. In summary, serotonin suppresses ethanol induced preference in two putative clusters IP and LP1. The reduced ethanol preference is due to prolonged serotonin signaling. The influence from IP and LP1 cluster on ethanol preference could be compromised by other serotonergic neurons which most likely the CSD neuron.

Appendix

Figure 1 *dSERT1* and *dSERT4* have same fragment size when use primers of L1345 and R2744.
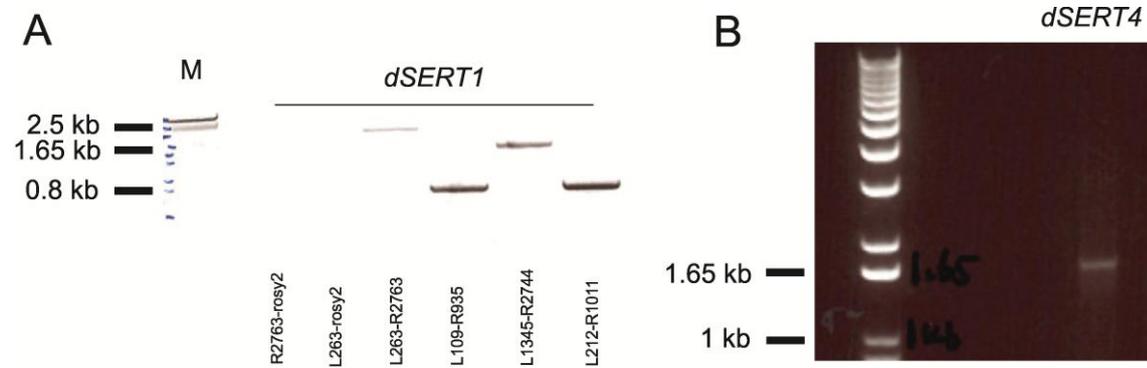
Figure 2 Wild type flies w^{1118} show decreased preference when raised in food with 0.8% of ethanol (nonparametric one sample sign test, a= different from random; * Student T-test, * $P < 0.05$).
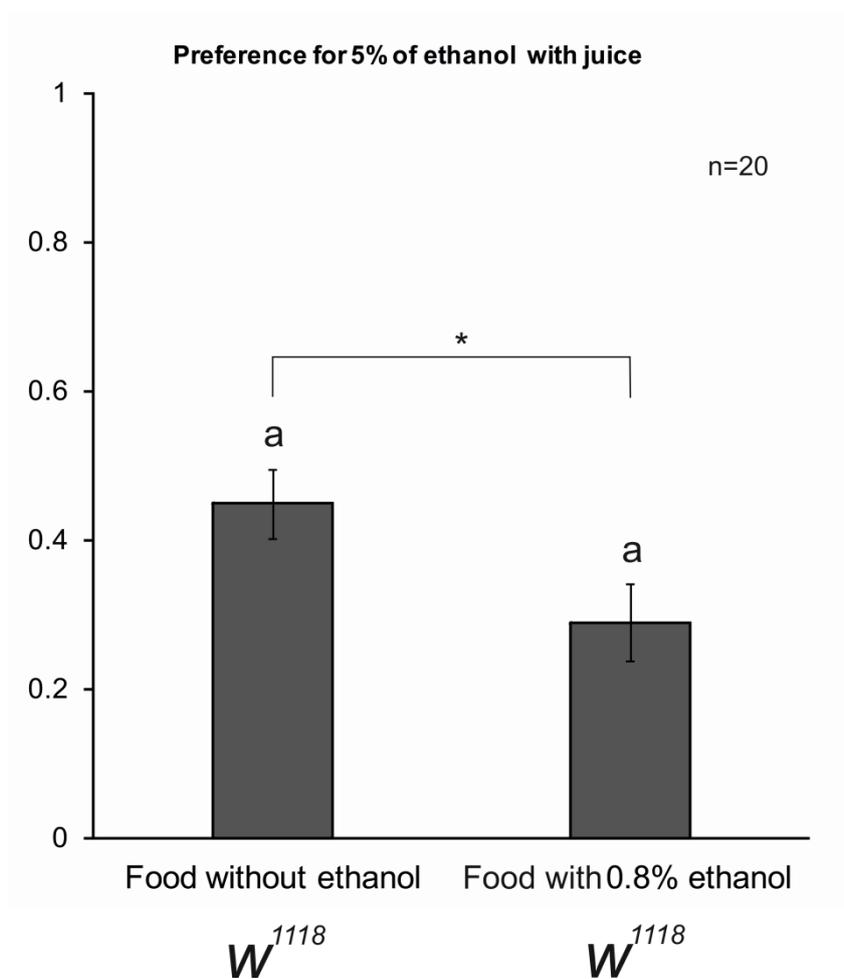
Figure 3 SERT3;RN2-E-GAL4/UAS-mCD8-GFP contains neurons from both SERT3 and RN2-E manner. (Arrows indicate the neurons driven by both SERT3-Gal4 and RN2-E-Gal4)
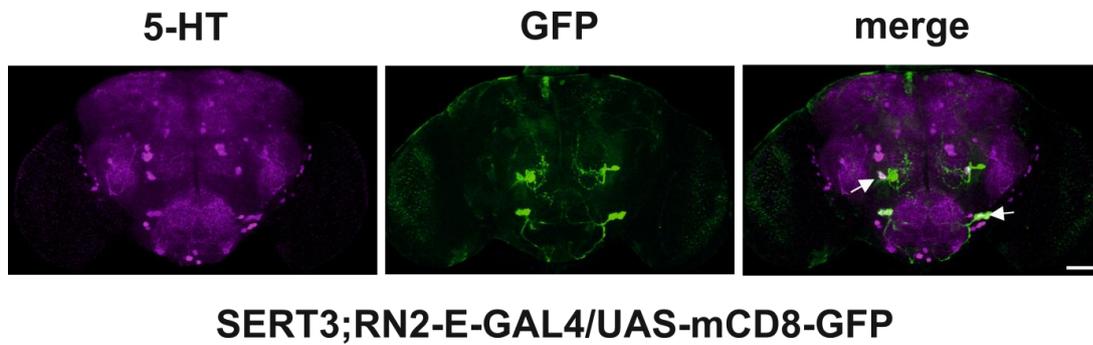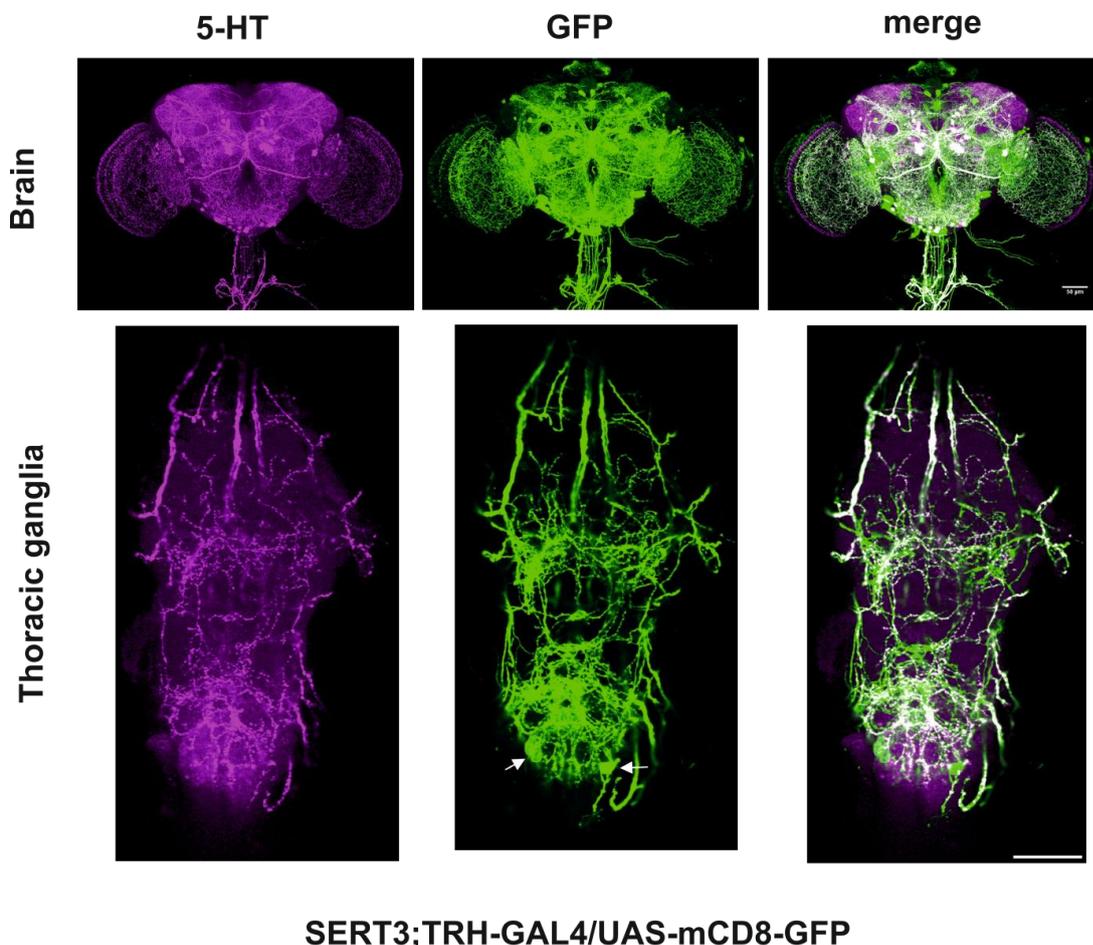

5-HT　　　　**GFP**　　　　**merge**

SERT3;RN2-E-GAL4/UAS-mCD8-GFP

Figure 4 SERT3;TRH-GAL4/UAS-mCD8-GFP contains neurons from both SERT3 and TRH manner. The unspecific neurons driven by SERT3-GAL4 showing in thorax with arrows.

5-HT　　　　**GFP**　　　　**merge**

Brain

Thoracic ganglia

SERT3;TRH-GAL4/UAS-mCD8-GFP

Abbreviations

5-HT	5-Hydroxytryptamin (Serotonin)
5HTR	serotonin receptor
ADH	Alcohol dehydrogenase
AM	Abdominal medial
AL	Antennal lobes
CC	Central Complex
Chr	chromosome
CNS	Central nervous system
DA	dopamine
Ddc	dopa decarboxylase
dSERT	*Drosophila* Serotonin Transporter
FB	Fan-shaped body
GFP	Green fluorescent protein
hSERT	human SERT
LN	Lateral neurons
MB	Mushroom body
MET	Mean elution time
rSERT	rat SERT
SD	Standard deviation
SEM	Standard error of the mean
SERT	Serotonin Transporter
SOG	Subesophageal ganglion
TDC	Tyrosine-decarboxylase
TPH	Tryptophan-hydroxylase
VMAT	vesicular monoamine transporter

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Acknowledgements

ERKLÄRUNG

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Li Xu