A Search for Neonicotinoid Binding Sites in the Optic Lobes of Honeybees and Blowflies

Author: Ashley Hulcoop, ashley.hulcoop.742@student.lu.se
Supervisors: Elisa Rigosi and David O'Carroll
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ABSTRACT

Neonicotinoid insecticides are agonists to nicotinic acetylcholine receptors (nAChRs) in the brains of insects. At sub-lethal doses these chemicals cause behavioral abnormalities in several bee species, reducing overall fitness. nAChRs expressed in the optic lobes of insects bind neonicotinoids, thus may be involved in these behavioral changes. Here we attempted without success to visualize the precise location of nicotinic cholinergic receptors together with choline acetyltransferase (ChAT) in the optic lobes of *Calliphora* sp. and *Apis mellifera* using immunohistochemistry techniques and the nAChR probe α-bungarotoxin.

INTRODUCTION

Insect pollination is economically and ecologically important, but populations are in decline due in part, to pesticide use (Kearns et al., 1998 Vanbergen et al., 2013; Hallmann et al., 2017). Pesticides are an important tool of modern agriculture for protecting against crop loss caused by pests such as aphids and whiteflies and neonicotinoids are one of the most widely used classes (Simon-Delso et al., 2015). Residual levels of neonicotinoids have been found in the environment, and in honey samples taken from around the world, including areas with restrictions on their use (Mitchell et al., 2017). These pesticide levels are concerning because there is evidence that neonicotinoids are harmful to both wild and domestic pollinators (Fryday et al., 2015).

Research shows that sub-lethal neonicotinoid levels affect behavior in a variety of bee species in the laboratory as well as in the field, with most behavioral research done on the honeybee, *Apis mellifera* (Fryday et al., 2015). Different neonicotinoids cause diverse changes in behavior. In one study, honeybees exposed to the neonicotinoid thiacloprid in the field had slower flight speeds and were much less likely to return to the hive compared to controls (Fischer et al., 2014). Several neonicotinoids including clothianidin, imidacloprid, and thiacloprid applied in doses close to what could be encountered by bees on foraging trips caused changes in homing navigation behavior due to a suspected effect on memory recall (Fischer et al., 2014). Lab experiments have shown that some of the pathways affected by neonicotinoids are involved in long term memory formation and olfactory learning (Gauthier et al., 2006; Dupuis et al., 2012). These pathways are found within the antennal lobes and mushroom bodies, areas of the brain that receive and process information (Barbara et al., 2008; Palmer et al., 2013). nAChRs are also found in the optic lobes of diverse insects including the fruit fly, honeybee, and blowfly (Brotz and Borst 1996; Chamaon et al., 2000; Thany et al., 2005; Thany et al., 2010).

Effects of neonicotinoids on the optic lobes has not been the focus of as much research as the antennal lobes and mushroom bodies. In one systematic literature review of neonicotinoid research including literature from 1990 to 2014, there were only two similar studies focused on optic lobes of bees

(Fryday et al., 2015). They found that imidacloprid and thiamethoxam applied to Africanized honeybee brains in sub-lethal doses caused negative changes in cell morphology to cells in the optic lobes at lower doses and exposure times than for cells in the mushroom bodies (Almeida Rossi et al., 2013; Oliviera et al., 2014). The higher sensitivity of the optic lobes to these neonicotinoids begs further investigation.

Neonicotinoids target nAChRs, which are ion channels on post-synaptic neurons in insects and vertebrates. They are located throughout the insect nervous system and compared to vertebrate nAChRs, have a higher affinity for neonicotinoids (Matsuda et al., 1998). The neurotransmitter acetylcholine binds to a site on the nicotinic acetylcholine receptor, which causes the ion channel to open and cations to enter. The flood of cations results in depolarization and excitement of the neuron (Reviewed in Breer, et al., 1987). Compounds in the neonicotinoid family are full or partial agonists to nicotinic acetylcholine receptors (Matsuda et al., 1998), which means they act in a similar way to the native ligand. Studies have shown that nAChRs consist of different subunits and isoforms that combine to create receptors with different affinities for ligands in bees and fruit flies (Matsuda et al., 1998, Matsuda et al., 2000; Sattelle et al., 2005; Jones et al., 2006, Barbara et al., 2008). This variation is one reason why sensitivity to the variety of neonicotinoid substances varies across species and brain regions.

The visual system of flying insects is highly complex, involving form, colour and motion processing (Brotz and Borst, 1996; Brotz 2001; Synakavitch and Strausfeld, 2004), so an effect on the neurons of the optic lobe might be one explanation for the decreased fitness of certain insects after exposure to neonicotinoids.

Previous studies have located and visualized either nAChRs or choline acetyltranferase (ChAT) to see cholinergic pathways (Thany et al., 2005; Thany et al., 2010). These studies found nAChR between the lamina and medulla of the optic lobes of honeybees. The goal of this experiment was to visualize nicotinic acetylcholine receptors and places of acetylcholine production together in the optic lobes of *Calliphora* sp. (Diptera) and *Apis mellifera* (Hymenoptera). We would expect to see nAChR and ChAT immunofluorescence located together, but not overlapping at pre- and post-synaptic junctions. Double labelling with both ChAT and α-BGT would provide better evidence for the presence of nAChRs than using α-BGT alone because if they appear as expected, it is less likely that the antibody binding was random or nonspecific.

To locate and visualize nAChRs and ChAT, we used immunohistochemistry (IHC) techniques and probes. IHC is a technique that uses the specificity of antibodies for their antigen to locate molecules in tissue samples (Kalyuzhny 2016). We indirectly visualized nAChRs, using alpha bungarotoxin (α-BGT), a probe conjugated to biotin. Biotin binds to a streptavidin containing fluorophore, which allows for visualization of the α-BGT binding location. To locate ChAT, we used an anti-ChAT antibody together with a secondary antibody plus fluorophore. Secondary antibodies or probes contain regions that recognize and bind specifically to the primary antibody or probe and are also conjugated to a fluorophore, a molecule that emits light when excited at a specific wavelength (Kalyuzhny 2016).

α-BGT is a useful compound for the identification and analysis of nAChRs in the nervous systems of various insects (Huang and Knowles 1990). It is an acetylcholine receptor antagonist that binds nAChRs in a similar way to neonicotinoids under certain conditions (Schmidt Nielsen et al., 1977).

Neonicotinoids have been found to displace radiolabelled α-BGT from nAChRs showing that biotinylated α-BGT together with a secondary fluorophore can be used to locate the specific receptors involved in neonicotinoid insect toxicity (Buckingham et al., 1995; Matsuda et al., 2001).

Acetylcholine is assembled by choline acetyltransferase (ChAT) at the pre-synaptic neuron before it enters the cleft via vesicle. Antibody labelling of this enzyme is a commonly used technique for the indirect visualization of cholinergic pathways (Thany et al., 2010).

We double-labelled images with primary antibodies that bind common brain structures to give context to nAChRs and ChAT binding. Anti-synapsin I binds a synaptic vesicle protein visualizing neuropiles (Watson et al., 2002), and anti-α-tubulin labels a component of the cytoskeleton present throughout cells, including along the length of axons and dendrites in neurons (Carceres et al., 1986). We also used the probe phalloidin conjugated to a fluorophore to label actin filaments, another component of the cytoskeleton.

Immunolabelling can be tricky as there are many factors that can affect the outcome. There are established protocols for tissue preparation for antibody labelling and immunofluorescence imaging, but there are many variables (E.g. Kreissel and Bicker, 1989; Ott, 2008; Chen et al., 2010; Howatt and Wilson, 2014). Fixation and permeabilization varies in which compounds and concentrations are used, duration of steps and whole brain versus sections. The protocols used here were based off previous experiments done by others using whole brains, even though we used sections. All these steps combine to hopefully produce sections with clear and specific patterns of staining when viewed under a laser scanning confocal microscope or fluorescence microscope.

MATERIAL AND METHODS

We anesthetized laboratory raised male and female blowflies (*Calliphora* sp.) and wild caught female worker honeybees (*Apis mellifera*) of unknown age on ice and decapitated them. Alternatively, the head cavity of the live insect was opened and 1.25 μM of α-BGT was applied. The insect was left for 90 minutes, the head capsule was washed with ringer solution (NaCl 140 mM; $NaHPO_4$ 4 mM; Na_2HPO_4 6 mM; Sucrose 90 mM; KCl 10 mM; $CaCl_2$ 2 mM and NaOH or HCl to pH 6.8 in MQ water) continuously for 5 minutes and the head was removed. We attached the heads to beeswax, opened the head capsule and applied ringer to cover. Non-brain tissue was removed, but some tracheae and retina remained. Brains were isolated and placed in fixative. Dissection lasted an average of 33 minutes.

We used two different methods of fixation. To half we added 4 % paraformaldehyde (PFA) and the other half zinc-formaldehyde (Zn-FA; Ott, 2008). They were left at 4˚ C overnight sealed with parafilm.

We washed Zn-FA fixed brains in tris buffer solution (TBS; 50 mM Tris base; 150 mM NaCl; pH adjusted to 7.5 with HCl) and PFA fixed brains in phosphate buffer solution (PBS; pH 7.4) 3 x 20 minutes at room temperature (RT) on a shaker. All washing and incubation for the duration of the experiment was performed in the same conditions. We placed brains into molds with gelatin albumin and added fixative. Several of the specimens first fixed in Zn-FA were then fixed in PFA but we continued to use TBS on those specimens for the duration of the experiment. We left specimens overnight at RT for PFA and at 4˚ C for Zn-FA. We washed brains in designated buffer 3 x 20 minutes,

preserved specimens with sodium azide (1:20 NaN_3:PBS / TBS) and stored them at 4˚ C for 3 – 4 days.

We washed brains in PBS / TBS 3 x 20 minutes, sectioned them horizontally (100 µm) using a vibratome and placed slices in multi-well plates in buffer. The central 5 or 6 sections of each brain we kept for primary antibody staining, distributed odd and even across two wells. The outer layers we kept for no antibody or secondary antibody only controls in different wells. Plates were left at 4˚ C overnight.

We washed sections 6 x 20 minutes with designated buffer plus either 1 or 0.5 % Triton X-100 (TX100) and incubated for 2 – 3 hours with pre-blocking solution (PBS / TBS + 5 % normal goat serum (Life Technologies) and either 1 or 0.5 % Triton X-100). In low light, we added primary antibody / probe solution (PBS / TBS with 1 % normal goat serum and either 1 or 0.5 % Triton X-100 + antibodies / probes) and left at 4˚ C under aluminum foil for 5 – 6 days. Sections were flipped once during that time. Antibodies / probes were combined with the antibody base solution as follows:

- Alexa Fluor 647 Phalloidin 1:50 (Excitation at 650, emission at 668; ThermoFisher) + α-tubulin mouse monoclonal antibody 1:500 or 1:100 (ThermoFisher)
- Alexa Fluor 647 Phalloidin 1:50 + α-bungarotoxin Biotin – XX 1:100 (ThermoFisher)
- Alexa Fluor 647 Phalloidin 1:50 + Choline acetyltransferase mouse monoclonal antibody (DSHB Hybridoma Product ChAT4B1) 1:500 or 1:100
- α-bungarotoxin Biotin – XX 1:100 + α-tubulin mouse monoclonal antibody 1:500 or 1:100
- α-bungarotoxin Biotin – XX 1:100 + Choline acetyltransferase mouse monoclonal antibody 1:500 or 1:100
- α-bungarotoxin Biotin – XX 1:100 + Anti-synapsin mouse monoclonal antibody (DSHB Hybridoma Product 3C11 (anti SYNORF1)) 1:50
- Anti-synapsin mouse monoclonal antibody 1:50 + Choline acetyltransferase mouse monoclonal antibody 1:500 or 1:100

We washed sections 6 x 20 minutes in PBS / TBS buffer and either 1 or 0.5 % TX100 then added secondary antibody solutions (PBS / TBS with 1 % normal goat serum and either 1 or 0.5 % Triton X-100 + polyclonal secondary antibodies from ThermoFisher Scientific) based on primary antibodies (Table 1). Sections were incubated for 3 days at 4˚ C and flipped once in that time.

Table 1: Secondary (2˚) antibodies or probes with fluorophores were combined with primary (1˚) antibodies to allow for the use of different channels during laser confocal microscopy.

2˚ **Antibody / Probe**	**2˚ Antibody Concentration**	**Excitation / Emission (nm)**	**1˚ Antibody / Probe**
Goat anti-Mouse IgG with Cy3	1:300	488 / 565	Anti-synapsin, ChAT, or α-Tubulin
Streptavidin – Cy3	1:500	488 / 565	Alpha bungarotoxin Biotin – XX
Streptavidin – Cy5	1:500	600 – 650 / 670	Alpha bungarotoxin Biotin – XX
Alexa Fluor 647 Goat anti – mouse	1:300	650 / 668	Synapsin, ChAT

We washed sections in PBS / TBS plus TX100 (1 or 0.5 %) 6 x 20 minutes then dehydrated and rehydrated sections with ascending then descending concentrations of ethanol for 15 minutes each (50, 60, 70, 80, 90, 95 and 100 %) with 5 minutes in a vacuum chamber during the 100 % ethanol rinse. We washed sections in PBS / TBS 2 x 20 minutes then left them overnight at 4˚ C or mounted them immediately using RapiClear 1.52 clearing medium on glass slides in spacer wells.

Sections were pre-screened with a fluorescence microscope based on quality of mounting and level of fluorescence. The best sections were imaged using a Zeiss LSM 510 META confocal microscope or a Leica SP8 DLS confocal microscope. Images were edited using Fiji (ImageJ).

RESULTS

We were unable to obtain quality images for each combination of conditions. Anti-synapsin labelling shows the location of several brain regions including the structures of the optic lobes (Fig 1; TX100 0.5 % + PFA fix). Double-labelling under these conditions using synapsin staining as a background for α-BGT gave no specific staining of nicotinic acetylcholine receptors by α-BGT (Fig. 2A). Instead α-BGT or the secondary probe streptavidin - Cy3 bound to structures outside the neuropiles. Phalloidin staining failed under all conditions that we tried and we observed only a faint uniform signal. Double-labelling with phalloidin + anti-ChAT (PFA fix, 0.5 % TX100) can be seen in the composite image Fig. 2B. Anti-ChAT gave a similar uniformly weak signal and showed no specific binding to choline acetyltransferase. α-BGT and anti-ChAT did not colocali ze when fixed in PFA and treated with 1 % TX100 (Fig. 2C). Instead, we saw a similar pattern of staining to that of Fig. 2A. Besides a different combination of antibodies, the detergent concentration between A (0.5 % TX100) and C (1 % TX100) was also different, but differences in staining strength or specificity were not noticed. An α-tubulin + α-BGT treated sample (Zn-FA fix with 0.5 % TX100) showed labelling for α-tubulin in the inner chiasma, the inner medulla and lobula (Fig. 2D, red). There was no specific labelling by α-BGT or by the fluorophore bound to α-BGT (Fig. 2D, green). Anti-synapsin labelling in similarly treated samples gave different results ranging from very strong (Fig 1 and 2A) to no specific labelling. Synapsin staining in the bee brain resulted in no labelling of synaptic structures and only a faint outline of the medulla and lobula complex (Fig. 3B; PFA fix, 1 % TX100).

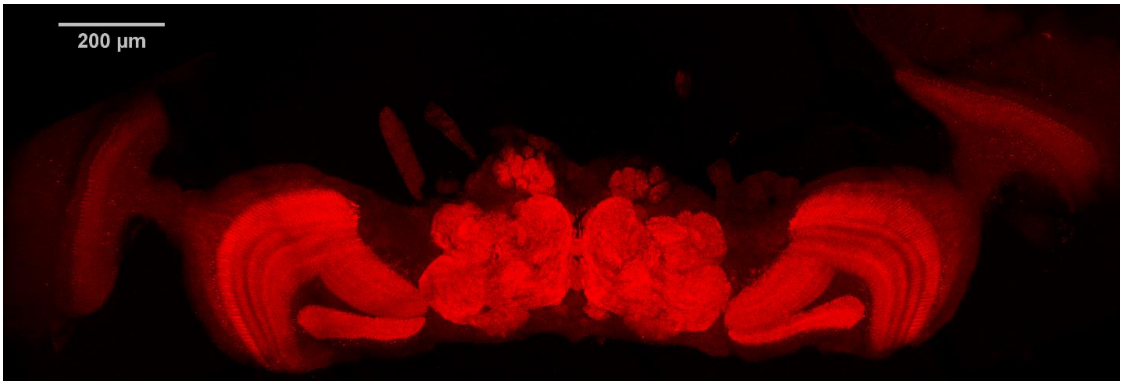

Fig. 1 Immunoreactivity of anti-synapsin in a 100 µm thick horizontal section of *Calliphora* sp. brain treated with 0.5 % TX100 + PFA fix. Image was obtained with a Leica SP8 DLS confocal microscope (excitation at 488 nm, emission at 565 nm).

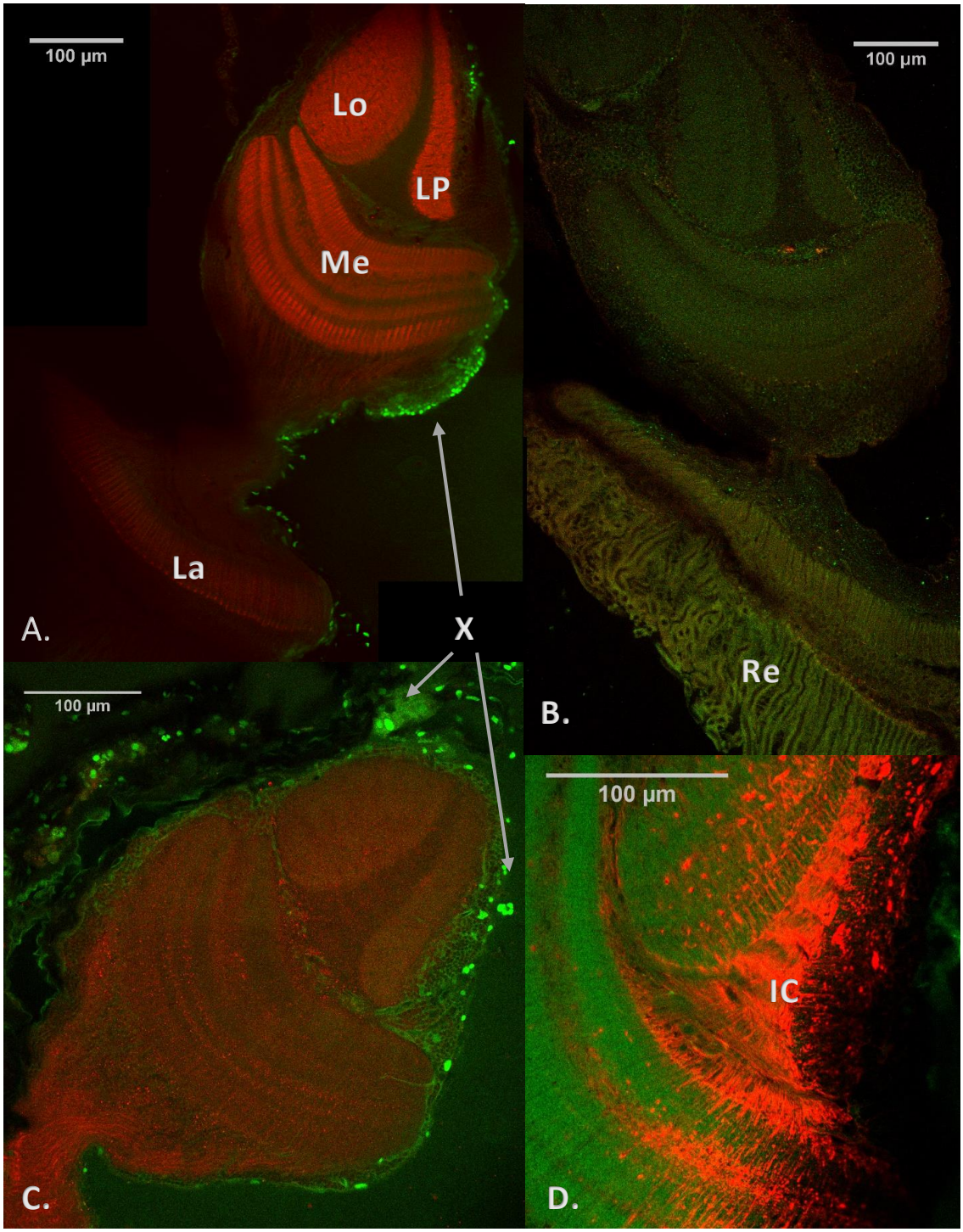

Fig. 2 A – D. Merged channel images of double-labelled *Calliphora* sp. optic lobes. **A**: anti-synapsin staining (red) showing the lamina (La), medulla (Me), lobula (Lo), and lobula plate (LP) overlaid with the green channel emitting maximally at 565 nm. X refers to similar looking staining in A & C. Treated with TX100 0.5 % + PFA fix. **B**: Faint phalloidin staining (red) likely autofluorescence masked by anti-ChAT staining (green) except at lamina and retina (Re) where fluorescence levels of both channels overlap (yellow). Treated with TX100 0.5 % + PFA fix. **C**: anti-ChAT (red) and α-BGT (green) with a similar pattern of staining compared to A. Treated with TX100 1 % + PFA fix. **D:** α-Tubulin (red) immunoreactivity in the inner chiasma (IC) and surrounding neuropiles of medulla and lobula. Treated with TX100 0.5 % + Zn-FA fix.

In the same brain, α-BGT did not specifically label nAChR and also did not show the same staining pattern as for 2A and C. Both examples treated with PFA + TX100 0.5 % together with anti-synapsin showed at least some or a good amount of synapsin staining (Fig. 2A, 4C & D). An anti-synapsin + α-BGT labelled section (0.5 % TX100 + PFA fix) viewed from the side showed a gradient of fluorescence intensity from the outside in for synapsin (Fig. 4D) and no gradient for α-BGT (Fig. 4B).

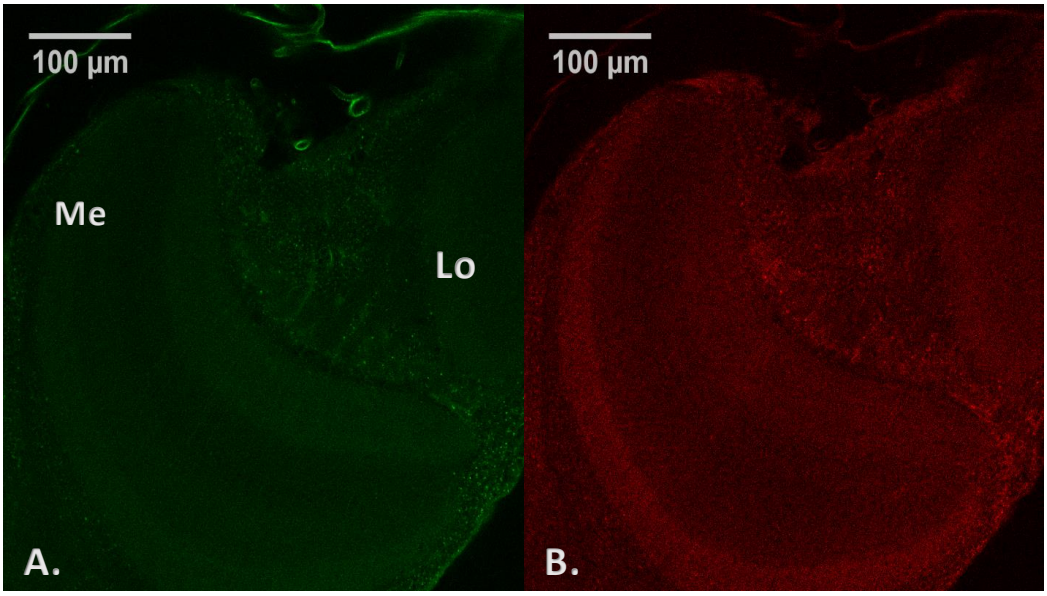

Fig. 3 Split channel of the optic lobe of a bee double labelled with α-BGT **(A)** and anti-synapsin **(B)** in 1 % TX100 + PFA fix. Neither nAChR or synapsin are specifically labelled.

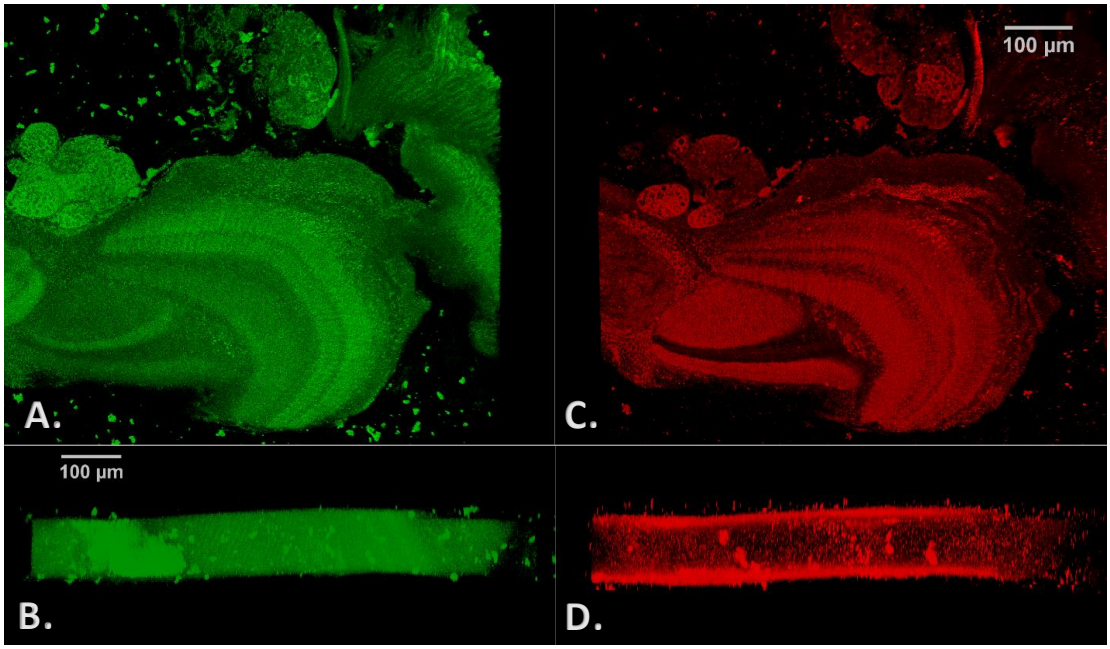

Fig. 4, A – D: Single 100 µm thick section of *Calliphora* sp. optic lobe treated with 0.5 % TX100 + PFA fix and exposed to α-BGT + streptavidin – Cy3 fluorophore and anti-synapsin + GaM – AF647 secondary antibody. **A & B**: Non-specific α-BGT or streptavidin – Cy3 labelling, or autofluorescence. **C & D**: Anti-synapsin staining. **B & D**: Split channel of brain exposed to α-BGT (B) and synapsin (D) showing even intensity throughout for α-BGT and different fluorescence intensity at different levels for anti-synapsin.

There were no noticeable differences between sections from insects treated with α-BGT before or after death.

A comparison between α-BGT treated (same section as Fig. 4) and an untreated control taken at the same settings and using the same wavelength showed a similar pattern and intensity of fluorescence (Fig. 5A & B). Overall, differences between detergent concentrations, fixative, and timing of α-BGT exposure were not noticed.

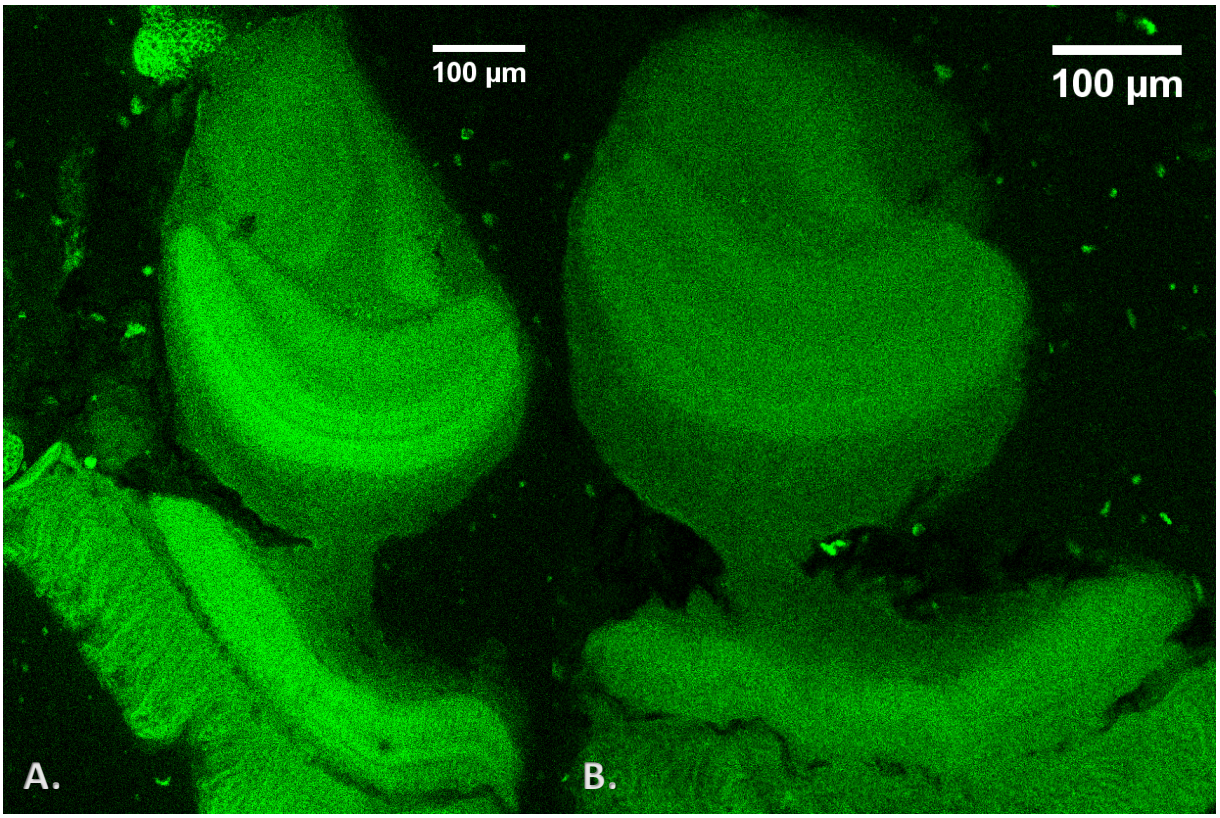

Fig. 5 100 µm thick *Calliphora* sp. sections showing optic lobes at different levels. A: treated with αBGT and Str-Cy3 B: Control not treated with probes, but final washing, dehydration and rehydration was like A.

DISCUSSION

The methods employed in this study did not work. We failed to label nAChRs and ChAT separately, or together in the same sample. We know based on previous studies, that nAChRs exist in the optic lobes of flies and bees, and that it is possible to label them with α-BGT or anti-ChAT (Buchner et al., 1986; Kreissl and Bicker, 1989) although examples of double labelling could not be found.

Expected α-BGT labelling patterns include a layer in the lamina (c-layer in bees; Kreissl and Bicker, 1989) or the outer layer of the lamina close to the retina (locust; thany et al., 2010). More generally α-BGT sensitive binding regions have been found throughout the lamina, medulla, lobula and lobula plate of the optic lobe (fruit fly; Chamaon et al., 2000). Expected ChAT localization is throughout the optic lobe neuropiles, but especially within the medulla and the outer layer of the lobula (fly;

Sinakevitch and Strausfeld, 2004; locusts, Thany et al., 2010). We did not see any of this. If we had, then signals would be very close to one another, but not overlapping at high enough resolution, signifying a pre- and post-synaptic cholinergic neuron.

Counter staining with synapsin or phalloidin is a commonly used technique with a long history of success, but that also gave inconsistent results. This inconsistency points to our method as the source of failure. One potential issue is that our protocols were based on whole brains, whereas we used 100 um thick sections. The consequence would be an overly intense treatment, including excessive washing, and permeablization. Existing methods that deal with sections have shorter fixation, washing and incubation times, and use a lower TX100 concentration (Buchner et al., 1986; Kreissl and Bicker 1989).

The antibody manufacturers (DSHB & ThermoFischer Scientific) suggest lightly centrifuging the antibodies before use and taking only the supernatant to avoid including peptides that may cause non-specific labelling. They also suggest that antibodies stored at 4˚ C should be used within two weeks. We did neither of these things. Poor mixing of the antibodies could be a factor in failed staining, but we noticed that there was poor penetration into the slice by both anti-α-tubulin and anti-synapsin (Fig. 2D & 4D), instead suggesting a problem with permeability. Ott (2008) proposes that a Zn-FA provides a better result for penetration of synapsin, but our best synapsin result (Fig 1 & 2A) was fixed in FA, so this appears to not be a main reason. Other factors involved could be the detergent concentration.

Images mostly showed what appears to be autofluorescence, which is indicated by the similarity of Figs. 5 A and B. Both images, taken using the same settings, show the α-BGT channel (excitation 488 nm, emission 565 nm), but while A was treated with α-BGT, B is entirely free from antibodies. A slightly stronger signal in A may be attributed to non-specific binding of the secondary antibody. The only bee brain imaged (Fig. 3) likely also shows autofluorescence as opposed to any kind of immunofluorescence, since the signal is uniform and weak. In the case of Figs. 2A & C, α-BGT staining produced a specific looking signal that appears to coincide with the known location of cell bodies or glia (Nässel et al., 1988). This signal is likely a false positive non-specific labelling by the secondary antibody, since this is a common problem in IHC (Burry, 2011) but as we have no type of control for those sections, it is not possible to conclude anything about it. If repeated, several things about the experiment would need to be modified. For example, controls for each scenario would be needed to make reliable assumptions about fluorescence images. Here, we only made a few.

The risks to pollinators of several neonicotinoids have been well established and restrictions for their use in agriculture are being implemented. These restrictions of the more widely used neonicotinoids (imidacloprid, clothianidin, and thiamethoxam) may cause an increase in the use of other neonicotinoids. For example in 2016, the maximum residue level for thiacloprid in honey was raised in the EU. However, in field tests, honeybees treated with thiacloprid had noticeaby slower flight speed compared to those exposed to imidacloprid, clothianidin and control (Fischer et al., 2014). It is clear that these pesticides affect various insect species and brain regions in different ways and that more studies are needed to elucidate the hazards to pollinators.

REFERENCES

Barbara, G.S., Grünewald, B., Paute, S., Gauthier, M., Raymond-Delpech, V. 2008. Study of nicotinic acetylcholine receptors on cultured antennal lobe neurones from adult honeybee brains. Invertebr. Neurosci. 8: 19–29. https://doi.org/10.1007/s10158-007-0062-2

Breer, H., Sattelle, D. 1987. Molecular properties and functions of insect acetylcholine receptors. J. Insect Physiol. 33: 771–790. https://doi.org/10.1016/0022-1910(87)90025-4

Brotz, T.M., Borst, A. 1996. Cholinergic and GABAergic receptors on fly tangential cells and their role in visual motion detection. J. Neurophysiol. 76: 1786–1799. https://doi.org/10.1016/0006-8993(90)90325-6

Brotz, T.M., Gundelfinger, E.D., Borst, A. 2001. Cholinergic and GABAergic pathways in fly motion vision. BMC Neurosci. 2. https://doi.org/10.1186/1471-2202-2-1

Buckingham, S.D., Balk, M.L., Lummis, S.C., Jewess, P., Sattelle, D.B. 1995. Actions of nitromethylenes on an alpha-bungarotoxin-sensitive neuronal nicotinic acetylcholine receptor. Neuropharmacology 34: 591–597. https://doi.org/002839089500024Z [pii]

Buchner, E., Buchner, S., Crawford, G., Mason, W.T., Salvaterra, P.M., Sattelle, D.B. 1986. Choline acetyltransferase-like immunoreactivity in the brain of Drosophila melanogaster. Cell Tissue Res. 246: 57 – 62

Burry, R. W. 2011. Controls for Immunocytochemistry: An Update. J Histochem Cytochem, 59: 6–12. http://doi.org/10.1369/jhc.2010.956920

Caceres, A., Banker, G.A., Binder, L. 1986. lmmunocytochemical Localization of Tubulin and Microtubule-Associated Protein 2 During the Development of Hippocampal Neurons in Culture. J. Neuroscience 6: 714–722.

Chen, X., Cho, D. and Yang, P. 2010. Double staining immunohistochemistry. N Am J Med Sci. 2: 241–245.

De Almeida Rossi, C., Roat, T.C., Tavares, D.A., Cintra-Socolowski, P., Malaspina, O. 2013. Brain morphophysiology of Africanized bee Apis mellifera exposed to sublethal doses of imidacloprid. Arch. Environ. Contam. Toxicol. 65: 234–243. https://doi.org/10.1007/s00244-013-9897-1

Dupuis, J., Louis, T., Gauthier, M., Raymond, V., 2012. Insights from honeybee (Apis mellifera) and fly (Drosophila melanogaster) nicotinic acetylcholine receptors: From genes to behavioral functions. Neurosci. Biobehav. Rev. 36: 1553–1564. https://doi.org/10.1016/j.neubiorev.2012.04.003

Fischer, J., Müller, T., Spatz, A.K., Greggers, U., Grünewald, B., Menzel, R., 2014. Neonicotinoids interfere with specific components of navigation in honeybees. PLoS One 9. https://doi.org/10.1371/journal.pone.0091364

Fryday, S., Tiede, K., Stein, J. 2015. Scientific services to support EFSA systematic reviews: lot 5 systematic literature review on the neonicotinoids (namely active substances clothianidin, thiamethoxam and imidacloprid) and the risks to bees. EFSA J. EN-756: 656.

Gauthier, M., Dacher, M., Thany, S.H., Niggebrügge, C., Déglise, P., Kljucevic, P., Armengaud, C., Grünewald, B., 2006. Involvement of α-bungarotoxin-sensitive nicotinic receptors in long-term memory formation in the honeybee (Apis mellifera). Neurobiol. Learn. Mem. 86: 164–174. https://doi.org/10.1016/j.nlm.2006.02.003

Howat, W.J. and Wilson, B.A. 2014. Tissue fixation and the effect of molecular fixatives on downstream staining procedures. Methods. 70: 12 – 19

Huang, Z.Y., Knowles, C.O., 1990. Nicotinic and muscarinic cholinergic receptors in honeybee brain. Comp. Biochem. Physiol. 97: 275 – 281

Hallmann C.A., Sorg M., Jongejans E., Siepel H., Hofland N. 2017. More than 75 percent decline over 27 years in total flying insect biomass in protected areas. PLOS ONE 12: e0185809 https://doi.org/10.1371/journal.pone.0185809

Jones, A.K., Raymond-delpech, V., Thany, S.H., Gauthier, M., Sattelle, D.B. 2006. The nicotinic acetylcholine receptor gene family of the honey bee, Apis mellifera. Genome Res. 16: 1422–1430. https://doi.org/10.1101/gr.4549206.

Kalyuzhny, A.E. 2016. Techniques in Life Science and Biomedicine for the Non-Expert, Immunohistochemistry Essential Elements and Beyond. Springer International Publishing, Switzerland. 16 – 18 pp. https://doi.org/10.1007/978-3-319-30893-7

Kearns, C.A., Inouye, D.W., Waser, N.M. 1998. ENDANGERED MUTUALISMS: The Conservation of Plant-Pollinator Interactions. Annu. Rev. Ecol. Syst. 29: 83–112. https://doi.org/10.1146/annurev.ecolsys.29.1.83

Kreissl, S., Bicker, G. 1989. Histochemistry of acetylcholinesterase and immunocytochemistry of an acetylcholine receptor-like antigen in the brain of the honeybee. J. Comp. Neurol. 286: 71–84. https://doi.org/10.1002/cne.902860105

Matsuda, K., Buckingham, S.D., Freeman, J.C., Squire, M.D., Baylis, H.A., Sattelle, D.B. 1998. Effects of the α subunit on imidacloprid sensitivity of recombinant nicotinic acetylcholine receptors. Br. J. Pharmacol. 123: 518–524. https://doi.org/10.1038/sj.bjp.0701618

Matsuda, K., Shimomura, M., Kondo, Y., Ihara, M., Hashigami, K., Yoshida, N., Raymond, V., Mongan, N.P., Freeman, J.C., Komai, K., Sattelle, D.B. 2000. Role of loop D of the α7 nicotinic acetylcholine receptor in its interaction with the insecticide imidacloprid and related neonicotinoids. Br. J. Pharmacol. 130: 981–986. https://doi.org/10.1038/sj.bjp.0703374

Mitchell, E.A.D., Mulhauser, B., Mulot, M., Mutabazi, A., Glauser, G., Aebi, A. 2017. A worldwide survey of neonicotinoids in honey. Science. 358: 109–111. https://doi.org/10.1126/science.aan3684

Nässel, D.R, Ohlsson, L.G., Johansson, K.U.I., Grimmelikhuijzen, C.J.P. 1988. Light and electron microscopic immunocytochemistry of neurons in the blowfly optic lobe reacting with antisera to RFamide and FRMFamide. Neurosci J. 27: 347-362

Oliveira, R.A., Roat, T.C., Carvalho, S.M., Malaspina, O. 2012. Side effects of thiamethoxam on the brain and midgut of the africanized honeybee Apis ellifera (Hymenopptera: Apidae). Environ Toxicol. doi 10.1002/tox

Ott S.R. 2008. Confocal microscopy in large insect brains: Zinc–formaldehyde fixation improves synapsin immunostaining and preservation. J Neurosci Methods. 172: 220–230

Palmer, M.J., Moffat, C., Saranzewa, N., Harvey, J., Wright, G.A., Connolly, C.N. 2013. Cholinergic pesticides cause mushroom body neuronal inactivation in honeybees. Nat. Commun. 4: 1634–1638. https://doi.org/10.1038/ncomms2648

Sattelle, D.B., Jones, A.K., Sattelle, B.M., Matsuda, K., Reenan, R., Biggin, P.C. 2005. Edit, cut and paste in the nicotinic acetylcholine receptor gene family of Drosophila melanogaster. BioEssays 27: 366–376. https://doi.org/10.1002/bies.20207

Schulz, R., Bertrand, S., Chamaon, K., Smalla, K.H., Gundelfinger, E.D., Bertrand, D. 2000. Neuronal nicotinic acetylcholine receptors from Drosophila: two different types of alpha subunits coassemble within the same receptor complex. J. Neurochem. 74: 2537–46.

Simon-Delso, N., Amaral-Rogers, V., Belzunces, L.P., Bonmatin, J.M., Chagnon, M., Downs, C., Furlan, L., Gibbons, D.W., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D.P., Krupke, C.H., Liess, M., Long, E., Mcfield, M., Mineau, P., Mitchell, E.A., Morrissey, C.A., Noome, D.A., Pisa, L., Settele, J., Stark, J.D., Tapparo, A., Van Dyck, H., Van Praagh, J., Van Der Sluijs, J.P., Whitehorn, P.R., Wiemers, M. 2015. Systemic insecticides (Neonicotinoids and fipronil): Trends, uses, mode of action and metabolites. Environ. Sci. Pollut. Res. 22: 5–34. https://doi.org/10.1007/s11356-014-3470-y

Sinakevitch, I., Strausfeld, N.J. 2004. Chemical Neuroanatomy of the Fly's Movement Detection Pathway. J. Comp. Neurol. 468: 6–23. https://doi.org/10.1002/cne.10929

Schmidt-Nielsen, B.K., Gepner, J.I.,Teng, N.N.H., Hall, L.M. 1977. Characterization of an α-bungarotoxin binding component from *Drosophila melanogaster.* J. Neurochem. 29: 1013 – 1029

Thany S.H., Crozatier M., Raymond-Delpech V. Apisalpha2, apisalpha7-1 and apisalpha7-2: Three new neuronal nicotinic acetylcholine receptor alpha-subunits in the honeybee brain. Gene. 344:125-32

Thany, S.H., Tricoire-Leignel, H., Lapied, B. 2010. Identification of cholinergic synaptic transmission in the insect nervous system. Adv. Exp. Med. Biol. 683: 1–10. https://doi.org/10.1007/978-1-4419-6445-8_1

Vanbergen, A.J., Garratt, M.P., Vanbergen, A.J., Baude, M., Biesmeijer, J.C., Britton, N.F., Brown, M.J.F., Brown, M., Bryden, J., Budge, G.E., Bull, J.C., Carvell, C., Challinor, A.J., Connolly, C.N., Evans, D.J., Feil, E.J., Garratt, M.P., Greco, M.K., Heard, M.S., Jansen, V.A.A., Keeling, M.J., Kunin, W.E., Marris, G.C., Memmott, J., Murray, J.T., Nicolson, S.W., Osborne, J.L., Paxton, R.J., Pirk, C.W.W., Polce, C., Potts, S.G., Priest, N.K., Raine, N.E., Roberts, S., Ryabov, E. V., Shafir, S., Shirley, M.D.F., Simpson, S.J., Stevenson, P.C., Stone, G.N., Termansen, M., Wright, G.A. 2013. Threats to an ecosystem service: Pressures on pollinators. Front. Ecol. Environ. 11: 251–259. https://doi.org/10.1890/120126

Watson, A.H.D., Schürmann, F.W. 2002. Synaptic structure, distribution, and circuitry in the central nervous system of the locust and related insects. Microsc. Res. Tech. 56: 210–226. https://doi.org/10.1002/jemt.10031