Maj Institute of Pharmacology
Polish Academy of Sciences

prof. dr hab. Małgorzata Kajta
Laboratory of Neuropharmacology and Epigenetics
Department of Pharmacology
Smetna Street 12, 31-343 Krakow, Poland
e-mail: kajta@if-pan.krakow.pl

A REVIEW OF DOCTORAL DISSERTATION

Terence Al L. Abaquita

"The role of heme oxygenase in the nervous system of Drosophila melanogaster"

GENERAL CHARACTERISTICS OF THE DISSERTATION

The doctoral dissertation of Terence Al L. Abaquita was established under the supervision of Prof. Elżbieta Pyza. The research has been done at the Department of Cell Biology and Imaging of the Institute of Zoology and Biomedical Research; the Faculty of Biology of Jagiellonian University in Krakow. The doctoral project was developed in accordance with the Comparative INsect CHRONobiology (CINCHRON) project, which was funded by the European Union's Horizon 2020 Research and Innovation Programme under the Marie Skłodowska-Curie grant agreement number 765937.

The doctoral dissertation consists of two original papers, namely: Abaquita et al. (2021) *Antioxidants*: 10, 1716, and Abaquita et al. (2023) *Frontiers in Physiology*: 14, 1060175. The papers were published in renowned scientific journals, and their total impact factor, taking into account the year of publication, is 12.43. The PhD Student is the first author of both articles, which proves his leading role in their establishing.

The publications constituting the basis of the doctoral dissertation have been described in a 55-page study containing the following chapters: *List of figures*, *List of tables*, *List of supplementary materials*, *List of appendices*, *Summary in English and Polish*, *General introduction*, *Aims of the study*, *Results and discussion*, *Conclusions*, and *Bibliography* with a list of more than 300 publications that, according to the PhD Student, are the most important

in the context of his research. The conclusions were formulated in a clear and coherent manner, in direct relation to the research objectives and after a thorough analysis of the original results.

The description has been followed by the copies of the articles (Annexes 1 and 2) as well as appropriate supplementary materials and attachments containing, among others, declarations of authors' contribution to published manuscripts. The co-authors confirm the dominant role of the PhD Student in data collection and writing, which have been estimated at approximately 70% and 40%, respectively. The co-authors also point to his important (almost equal to the corresponding author) role in the analysis and interpretation of data, which was estimated at 30% in 1st manuscript, and 45% in 2nd manuscript.

SUBSTANTIVE ANALYSIS OF THE DISSERTATION

The reviewed doctoral dissertation focuses on 2 issues, which are:

1. Profiling of the gene encoding heme oxygenase i.e., *ho* in the brain of *Drosophila melanogaster*, with particular emphasis on:
    i. Response to the circadian clock (day, night, per^{01} mutant), light (LD12:12 or constant darkness), age (10-, 20-day-old), and oxidative conditions (paraquat *vs* curcumin).
    ii. Impact on DNA repair, measured by expression of *eIF4a* and *Xpc* genes.
    iii. Interactions with apoptosis and autophagy signaling (*hid*-, *skl*-, *atg5*-, *atg10*-related pathways). **Abaquita et al. (2021) *Antioxidants***
2. Identifying molecular mechanisms by which heme oxygenase affects degeneration and regulates apoptosis and autophagy in *Drosophila* flies, depending on:
    i. *ho* gene expression (*ho* overexpression or silencing in neurons),
    ii. Age (7-, 14-, 30-, 60-day-old),
    iii. Temperature (25 vs 29 °C),
    iv. Cell type (dopaminergic neurons, retina photoreceptors).

Abaquita et al. (2023) *Front Physiol*

As one can easily see, the PhD student carefully planned the consecutive stages of research and conducted them in a thoughtful and consistent manner. Particularly noteworthy is the vast theoretical knowledge presented in publications and their description, which found practical expression in the form of well-designed experiments. Their implementation took place thanks to the use of modern research methods and techniques, such as the GAL4/UAS system to

produce flies with upregulated or silenced *ho* expression, both in neurons and glial cells, molecular analyses focused on apoptosis-, autophagy- and DNA repair-related genes (*hid, skl, atg5, atg10, eIF4a, Xpc*), biochemical assessment of Dronc caspase activity, as well as immunostaining of dopaminergic neurons and retinal photoreceptors to visualize degenerations using scanning electron microscopy.

This wide range of research methods and techniques was skillfully used by the Doctoral Student to achieve the objectives of the dissertation, which were:

A. Delineating *ho* gene expression patterns in *Drosophila* brain under various conditions differing in circadian clock, access to light, age, and oxidative or heat stress.
B. Determining molecular mechanisms of interactive actions of heme oxygenase on DNA repair as well as on apoptosis and autophagy processes.
C. Assessment of the impact of neuronal heme oxygenase on survival and climbing behavior of *Drosophila*.

All the objectives were planned to provide a comprehensive description of the impact of heme oxygenase on the brain of *Drosophila melanogaster*.

I would like to emphasize here that, in addition to the high value in terms of basic research, the doctoral dissertation also has a certain translational value. Therapeutic potential of targeting the heme oxygenase enzyme has been reported. A randomized controlled trial confirmed the usefulness of the treatment with 5-aminolevulinic acid (5-ALA) with sodium ferrous citrate (SFC), known to act as a heme oxygenase-1 inducer, in preventing nephrotoxicity in patients undergoing cisplatin-based chemotherapy for lung cancer (Kawamura et al. 2022, *Oncology*). Clinical trial with the use of heme oxygenase inhibitor, tin mesoporphyrin, showed effectiveness in decreasing the total bilirubin level in newborns and reduced the severity of subsequent hyperbilirubinemia (Bhutani et al. 2016, *J Perinatol*). Heme oxygenase-1 polymorphism has been linked to the pathogenesis of preeclampsia (Lv X et al. 2020, *Clin Exp Hypertens*). Interestingly, heme oxygenase-1 which is targeted by HIV-1α subunit of hypoxia-inducible factor (HIV) has been postulated as a novel marker for anti-ischemic therapy in Raynaud syndrome (Heger et al. 2019, *Acta Pharmacol Sin*). Furthermore, patients planned for conventional aortic valve replacement who received heme arginate (heme oxygenase inducer) infused intravenously 24 hours before surgery showed a strong, dose-dependent increases in myocardial *HO-1* mRNA and atrial HO-1 protein levels (Andreas et al. 2018, *Arterioscler Thromb Vasc Biol*) that could prevent or reduce ischemia-reperfusion injury.

After reading the publications constituting the doctoral dissertation, I consider the most important achievements of the Author to be the demonstration that:

1. *ho* mRNA expression in *Drosophila* brain oscillates showing minimal values at the beginning of the day (ZT1) and night (ZT13). The daily rhythm of *ho* expression changes due to constant darkness or vanishes due to aging.
2. Exposure to paraquat increases the minimal expression levels of *ho* mRNA observed in *Drosophila* brain at ZT1 and ZT13. It also regulates expression levels of apoptotic *hid* and autophagic *atg5*; both *hid* and *atg5* are stimulated at ZT4, but *atg5* is inhibited at ZT1 and ZT13-ZT20.
3. Exposure to curcumin increases *ho* mRNA expression and restores its daily pattern in old/older i.e., 20-day-old animals. It also stimulates expression of apoptosis-related *hid* and *skl* at night (ZT13-ZT20), but inhibits expression of autophagy-related *atg5* and *atg10* during the whole cycle.
4. Overexpression of neuronal *ho*:
    i. Inhibits *Drosophila*'s survival and impairs climbing ability in older i.e., 14-day-old animals. Interestingly, *ho* silencing extends the survival approximately from 45 to 75 days and then inhibits it until 80 days, but impairs climbing ability as *ho* overexpression does.
    ii. Does not affect DNA repair in terms of *eIF4a* and *Xpc*. Also *ho* silencing does not affect it.
    iii. Causes a time-dependent (ZT16-specific) increase in expression of apoptotic *hid* and autophagic *atg5*.
    iv. Induces age-dependently, i.e., only in 7-day-old *Drosophila* fly heads, the apoptosis-related factors (*hid* expression and caspase Dronc activity) and the autophagy-related factors (*atg5* and *atg10* expression). Intriguingly, *hid* expression levels are similar in *ho* overexpressing and *ho* silenced flies.
    v. Evokes age-dependent, i.e., mainly in 7-day-old *Drosophila* flies, degeneration of dopaminergic neurons. Importantly, also *ho* silencing causes degeneration. For comparison, *ho* overexpression in retina photoreceptors causes degeneration in the proximal retina, without changes in the eye morphology.

5. Heat stress impairs climbing ability and *ho* expression but stimulates *hid* expression in *Drosophila* flies. Curcumin supplementation of heat-stressed animals improves survival and climbing ability as well as inhibits expression of *atg5* and *ho* which became dysregulated due to the curcumin treatment of non-stressed animals.
6. In *Drosophila* flies overexpressing *ho* in neurons, curcumin supplementation impairs survival and *hid* expression but does not affect climbing ability. In *Drosophila* flies with silenced *ho* in neurons, curcumin supplementation slightly improves survival and climbing ability but does not affect *hid* expression.

Given the above results, I have a few questions: **a.** Why all the experiments were performed on male *Drosophila* flies? **b.** How to explain the same expression levels of *hid* in heads of 7-day-old flies with up- and down-regulated expression of *ho*? Does it imply no interference between *ho* and *hid*? Fig. 2, *Front Physiol.* **c.** How to explain similar effects of paraquat and curcumin on expression of *ho*, *hid* and *atg5*? **d.** In the table 1 summarizing the role of *ho* in the brain, instead of actually used paraquat, rotenone has been included. Is this a mistake? **e.** Although apoptosis leads usually to cell death, autophagy may prevent cell death or stimulate it. Can you identify the role that heme oxygenase-associated autophagy plays in *Drosophila* brain?

SUMMARY AN FINAL CONCLUSIONS

Based on the above results, it can be suggested that profiles/patterns of *ho* expression in *Drosophila* brain undergo fluctuations due to circadian clock, access to light, aging as well as exposures to oxidative or heat stress. The study provided evidence for the interactive actions of heme oxygenase on apoptosis- and autophagy-related signaling, but not on DNA repair. Interestingly, both ROS inducer (paraquat) and ROS scavenger (curcumin) have abilities to stimulate expression of *ho* and apoptosis-related *hid* but inhibit autophagy-related *atg5*. Molecular mechanisms of heme oxygenase actions on *Drosophila* brain rely on age-dependent induction of the apoptosis- (*hid* expression and caspase Dronc activity) and autophagy-related factors (*atg5* and *atg10* expression) that correlates with neuronal degeneration. Functional analyses showed that curcumin supplementation may improve survival and climbing ability but only in heat-stressed or *ho* silenced *Drosophila* flies that involves the downregulation of *atg5* and *ho* but not *hid*.

As mentioned, the reviewed dissertation is a highly original basic study that was carried out on a model experimental animal, which allows for the *in vivo* implementation of modern, advanced

and sophisticated research approaches. Although apoptosis and autophagy are the basic processes underlying neuronal degeneration, which have become a challenge of civilization, knowledge about their interference with heme oxygenase in the *Drosophila* brain is surprisingly low. Considering the originality of the research undertaken and the use of modern methods and techniques in their implementation in the field of molecular biology, cell biology, biochemistry and SEM imaging, going beyond the usual requirements for doctoral theses, I recommend the doctoral dissertation for distinction.

Doctoral dissertation of Terence Al L. Abaquita, MSc, "*The role of heme oxygenase in the nervous system of Drosophila melanogaster*" meets the requirements of Article 187 of the Act of July 20, 2018 Law on Higher Education and Science (Journal of Laws of 2021, items 478, 619, 1630). I am applying to the Biological Sciences Discipline Council of the Jagiellonian University to admit Terence Al L. Abaquita, MSc, to the next stages of the doctoral procedure.

Rozprawa doktorska mgr. Terence'a Al L. Abaquita „*Rola oksygenazy hemowej w układzie nerwowym Drosophila melanogaster*" spełnia wymogi art. 187 ustawy z dnia 20 lipca 2018 r. Prawo o Szkolnictwie Wyższym i Nauce (Dz. U. z 2021 r.) , poz. 478, 619, 1630). Zwracam się do Rady Dyscypliny Nauk Biologicznych Uniwersytetu Jagiellońskiego o dopuszczenie mgr. Terence'a Al L. Abaquita do dalszych etapów przewodu doktorskiego.

Kraków, May 29th 2023

Małgorzata Kajta