EVALUATION REPORT FOR THE Ph.D. DISSERTATION OF SVITLANA ANTONIUK

Stress-related depressive disorders show a high prevalence worldwide and represent a major public health problem. Although currently available treatments, mainly based on specific serotonin reuptake inhibitors (SSRIs), show unequivocal efficiency, they require several weeks of treatment to observe a therapeutic benefit and around 30% of patients are resistant to any treatment. There is thus an urgent need for novel therapeutic strategies for the management of depression. In this context, the Ph.D. thesis of Mrs. Svitlana Antoniuk completed in the Laboratory of Cell Biophysics of the Nencki Institute of Experimental Biology under the co-supervision of Prof Jakub Wlodarczyk and Prof Evgeni Ponimaskin (Hannover Medical School) and exploring functional interactions between two serotonin receptor subtypes as a potential mechanism underlying stress-related depressive disorders, is without any doubt very timely and provides new insight into the etiology of these strongly debilitating pathologies.

The thesis is written in English, covers 87 pages and includes 220 external references. It contains 25 figures and 12 tables that are generally clear, relevant and informative. The title is adequate, linguistically precise and clearly reflects the work presented in the thesis. The abstract (one page and half, pages 8-9) clearly introduces the context, the thesis objectives and the strategy used, and provides a concise description of the main results presented in the manuscript.

The thesis dissertation classically begins with an introduction that describes the current knowledge on topics related to the thesis work. It includes five chapters dedicated to the presentation of serotonergic system and its role in depression, the description of serotonin receptor subtypes, with a special focus on 5-HT_{1A} and 5-HT_7 receptors (the subject matter of the Ph.D. thesis addresses) and their signal transduction properties, the functional cross-talks between both receptor subtypes and their impact upon neuronal signalling and, finally, animal models of depression. Overall, this introduction is concise (14 pages), clearly written, well-illustrated and pleasant to read. It is also well-documented and contains most the necessary information justifying the proposed experiments. Its shows that the candidate has a good knowledge of the subject. I have only a few minor comments on the introduction, as listed below:

- The candidate states on page 13 (last paragraph) that only the 5-HT_{1B} and 5-HT_{2B} receptors are crystallised, whereas the structure of the 5-HT_{2A} receptor complexed with a hallucinogen and Gαq has been reported in 2020 (Kim *et al.*, 2020, Cell 182, 1574–1588). In this line, she may have also quoted another recent Cell paper (Dong *et al.*, 2021, Cell 184, 2779–2792) showing how an engineered biosensor based on the 5-HT_{2A} receptor structure enabled the design of non-hallucinogenic ligands of the receptor producing rapid-onset and long-lasting antidepressant-like effects after a single administration.

- The serotonergic theory of depression may have been a bit tempered by mentioning data supporting the notion that a depressive state can be associated with decreased SERT density and/or 5-HT transport, such as the decrease in SERT function observed in some depressed patients and the greater risk for depression (associated to a decreased SERT expression) in individuals with the short serotonin transporter allele. These data suggest that depressive states are not systematically related to a deficit in 5-HT transmission.

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- Data supporting the role of presynaptic 5-HT_{1A} receptor in the delayed therapeutic action of SSRIs should have been mentioned in the introduction. Likewise, though briefly mentioned in the discussion, the antidepressant effects of 5-HT_{1A} and 5-HT_7 receptor antagonists in various depression paradigms, which are closely related to the experimental work realized by the candidat, should have been quoted in the introduction.

- The description of 5-HT_7 receptor constitutive activity on page 21 (4 last lines) is an important point that should have been placed before mentioning the effects of 5-HT_7 receptor inverse agonists on heterologous desensitization of other GPCRs (Krobert *et al.*, 2006). Furthermore, the effects of inverse agonists on receptor desensitization seem to be not correlated with their inverse agonist efficacy and the underlying mechanism remains to be elucidated. So, the interest of this study with respect of the thesis work is not obvious.

- The candidate describes on pages 22-23 the role of GPCR heteroreceptor complexes in neuropsychiatric disorders and considers complexes that do not implicate 5-HT receptors, such as the $A_{2A}/D_2/mGlu_5$ complex. She should have also mentioned the 5-$HT_{2A}/mGlu_2$ heterocomplex, which is considered as a major target of glutamatergic and serotonergic antipsychotics and has been involved in psychotic states.

The introduction is followed by the presentation of the three major objectives of the thesis, namely:

- Demonstrating the relevance of the chronic unpredictable stress to model depression in different rodent strains using a meta-analysis of literature data
- Determining the relative 5-HT_{1A} and 5-HT_7 receptor expression in various brain regions at different post-natal stages
- Determining the impact of chronic unpredictable stress upon 5-HT_{1A} and 5-HT_7 receptor heteromerization.

This is followed by a detailed “Materials and Methods” section describing the experimental procedures used by the candidate. This section, not always present in Ph.D. theses, is useful and appreciated, as it shows the strong implication of the candidate in her thesis work and her mastering of the methods used. The description of the protocols by the candidate generally provides sufficient details to reproduce the experiments. A strength of her thesis work is certainly its multi-disciplinarity and the combination of various experimental approaches.

The “Results” section begins with the description of the meta-analysis demonstrating the relevance of chronic unpredictable stress associated with the sucrose preference test (to assess anhedonic behaviour) in various rodent strains to model stress-associated depressive state. This study based on the reviewing of 485 published articles, led to the selection of 129 papers using various stress protocols of different duration. The data convincingly showed an anhedonic behaviour after applying a chronic unpredictable stress protocol in all rat and mouse strains and revealed that the different strains show different sensitivities to stress upon prolonging the chronic unpredictable stress. Based on the results of this meta-analysis, C57BL/6J mice were selected for further experiments as the best model due to their higher susceptibility to stress protocol of shorter duration in comparison to other strains and rats, and the availability of transgenic mouse lines with this genetic background. This meta-analysis yielded one article signed as first author by the candidate and published in Neurosci. Biobehav. Rev. in 2019.

Next, the candidate verified that an acute administration of the 5-HT receptor agonist 5-carboxamidotryptamine (5-CT) or the high-affinity 5-HT_7 receptor agonist LP-211 to C57BL/6J mice produces a depressive-like behaviour (increase in immobility time in the tail suspension test), corroborating previous results. Although the data are clear and convincing, the candidate cannot conclude from these results that 5-HT_{1A} and 5-HT_7 receptor stimulation leads to depressive phenotype in C57BL/6J mice (page 49, 3 last lines). Indeed, 5-CT is a non-selective receptor agonist capable of activating not only 5-HT_{1A} and 5-HT_7 receptors, but also 5-H_{1B} and 5-HT_{1D} receptors with high affinity and, given the dose administered (10 mg/kg), likely other 5-HT receptor subtypes, such as 5-HT_2 and 5-HT_6 receptors. A more exhaustive pharmacological study using a least a 5-HT_{1A} receptor antagonist and a 5-HT_7 receptor would definitely allow such a conclusion.

The candidate then explored the expression profile of 5-HT_{1A} and 5-HT_7 receptors at the transcript (assessed by qRT-PCR) and protein (assessed by Western blotting) levels at various post-natal stages (post-natal days 2, 12 and 90). The data clearly demonstrate co-expression of both receptors (though at different relative levels) in the three brain areas examined (prefrontal cortex, hippocampus and dorsal Raphe nuclei) and at all developmental stages, making possible the formation of 5-HT_{1A}/5-HT_7 receptor heterocomplexes. However, there are some discrepancies between qRT-PCR and Western blotting data that should be discussed during the thesis defence. One should keep in mind that Western blotting is a semi-quantitative method and that the results should be considered cautiously and ideally be confirmed by other more quantitative methods (e.g. radioligand binding experiments).

These experiments are followed by the most innovative part of the thesis dedicated to the impact of chronic unpredictable stress upon 5-HT_{1A}/5-HT_7 receptor heterodimerization, as assessed using the in-situ proximity ligation assay (PLA). The experimental strategy used is appropriate and the data convincingly demonstrate the formation of 5-HT_{1A}/5-HT_7 heterocomplexes, mostly in the PFC and, to a lesser extent, in the hippocampus and Raphe nuclei. Most importantly, heteromer formation was strongly decreased in prefrontal cortex of anhedonic animals, compared with control and resilient animals, whereas the expression of both receptors was unaffected. Likewise, 5-HT_{1A}/5-HT_7 receptor dimerization was decreased in the hippocampus (dendate gyrus) of stress-susceptible mice, compared to resilient mice, whereas no change in receptor heteromerization was found in Raphe nuclei, including in 5-HT neurons. Again, the data are convincing and promising and suggest that 5-HT_{1A}/5-HT_7 receptor heterodimerization might be an important mechanism contributing to the etiology of stress-related depressive states. I have just a small concern regarding the pattern of PLA staining depicted on Figure 20, which seems to be more scattered than receptor immunostainings illustrated on Figure 19. In fact, part of PLA staining seems to be outside the cells. This PLA staining pattern also differs from what was observed in human prefrontal cortex, where PLA blobs seem to be concentrated in the cell bodies (around nuclei, see ROI 2, Figure 21). How does the candidate explain these differences in PLA blob localization in the different experiments or samples? This point should also be discussed during the thesis defence.

The “Results” section is followed by a discussion of the biological significance of the data obtained by the candidate. Again, this section is well written, the comments are often pertinent and demonstrate her good knowledge of the subject. The dissertation ends with a short conclusion recapitulating the main results of the thesis and the reference list. I am only a bit concerned by the applicant’s conclusions regarding the relative receptor expression in the different brain structures due to the discrepancies of results at transcript and protein levels. Moreover, in absence of absolute quantification of receptor proteins, it is difficult to conclude on the actual proportion of both receptors in the different brain structures examined. Only the determination of receptor density thanks to

binding experiments would allow such a conclusion. In the same line of thinking, the impact of changes in 5-HT_{1A}-5-HT_7 receptor heterodimer concentration on the level of 5-HT_{1A} receptor homodimers and 5-HT_{1A} receptor coupling properties should be interpreted cautiously in absence of quantitative binding data on the relative densities of each receptor and the absence of information on the proportion of each receptor engaged in 5-HT_{1A}-5-HT_7 receptor heteromers.

Also, some references quoted in the discussion (page 71) and concerning the role of Gs signalling in antidepressant action are not in the Reference list (see for instance De Montis *et al.*, 1990; Menkes *et al.*, 1983; Ozawa and Rasenick, 1991).

In conclusion, this is nice piece of work, which represents an original contribution in the field of serotonin and mood disorders and provides new insight into the pathophysiological consequences of 5-HT receptor heteromerization. Therefore, I am convinced that the doctoral dissertation deserves the Ph.D. degree and this is without any doubt that I recommend to deliver the Ph.D. degree to Mrs. Svitlana Antoniuk.

Montpellier, February 3rd, 2022

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Kraków 9.02.2022 r.

Review of PhD thesis by Svitlana Antoniuk

Title: Interplay between serotonin 5-HT_{1A} and 5-HT_7 receptors in stress-related disorders.

Under supervisors: prof. Jakub Włodarczyk and prof. Evgeni Ponimaskin

Scientific merits of the thesis

The neurobiology of mood disorders has traditionally focused on monoamine neurotransmitters such as serotonin or norepinephrine. Based on the analysis of the basic antidepressant effect, which is inhibition of serotonin and/or norepinephrine reuptake, it is thought that reduction in synaptic levels of these aminergic neurotransmitters may be a major cause of depression. Since the discovery of current serotonin and noradrenergic antidepressants, there has been limited progress in developing new antidepressant treatments.

Therefore, the new concept that receptor heterodimerization is important for the action of aminergic neurotransmitters, such as serotonin, opens new approaches to understanding the mechanisms of neurotransmission as well as new strategies in drug development.

Ms. Antoniuk's PhD thesis focuses on a pair of two serotonin receptors, $5HT_{1A}$ and $5HT_7$. She attempted to explain the importance of heterodimerization of these receptors in the context of stress-related disorders, which undoubtedly include depression. Therefore, in her studies she used an animal model of chronic unpredictable stress-induced depression. Ms. Antoniuk approached the topic very professionally by first performing a theoretical study based on meta-analysis to verify the applicability of the chronic unpredictable stress protocol for modeling depression in different strains of rodents. After verifying the protocol, she continued research on the C57BL/6J mouse strain. Then she focused on studying the $5HT_{1A}$-$5HT_7$ receptor interaction. This is a very important aspect of research in pharmacology. The interaction between these types of receptors has been postulated for many years now and it has been suggested that it may have important pharmacological significance. In 2012, a publication of Prof. Ponimaskin's group - co-promoter of this dissertation - was published, in which they demonstrated the existence of $5HT_{1A}$-$5HT_7$ heterodimers by means of advanced biophysical methods, using the FRET technique. Having a solid basis for studying the dimerization of these receptors, Ms. Antoniuk determined, using the proximity ligation assay (PLA), the interaction of these receptors in the brains of C57BL/6J mice subjected to chronic unpredictable stress. She demonstrated a reduced number of tested heterocomplexes in anhedonic mice in prefrontal cortex, thus confirming that $5HT_{1A}$-$5HT_7$ complexes may have a functional role in the development of depression-like behavior.

Substantial merit of the thesis

The dissertation contains 88 typed pages, of which the first three pages are: *the title page, author's declaration, and acknowledgments*. After the *table of contents*, the author has included a *list of abbreviations*, followed by *abstracts in English and Polish*.

The introduction includes sections such as *Serotoninergic theory of depression*, *Serotoninergic system*, *5HT$_{1A}$ receptor*, *5HT$_7$ receptor*, *Functional crosstalk between 5HT$_{1A}$ and 5HT$_7$ receptors*, and the concluding subsection *Animal models of depression*. All subsections are written in a clear manner, well separated in terms of content in relation to the dissertation topic. At the beginning, Ms. Antoniuk describes in a very accessible way the role of serotonin in the etiopathogenesis of depression, proceeding to a detailed description of the types of serotonin receptors along with the pathways regulated by them. This description also deserves praise. It is written in a clear way without unnecessary descriptions, which is unfortunately common in many PhD theses. Next, the author goes into a detailed description of the 5HT$_{1A}$ and 5HT$_7$ receptor pair, which is the main object of her research. Especially interesting is subsection *Functional crosstalk between 5HT$_{1A}$ and 5HT$_7$ receptors*, in which she included the most important reports related to dimerization of GPCRs. In this subsection, not only did she focus on the studied pair of receptors, but also extended the subsection to include additional receptor pairs that form functional dimers, which may have an impact on neurological disorders. Thus, the role of adenosine receptors and their effect on dopamine D$_1$ and D$_2$ receptors in Parkinson's disease was highlighted. As the PhD thesis concerns the involvement of receptor dimers in stress-related disorders, it is therefore unfortunate that information on D$_1$-D$_2$ in the context of depression was lacking.

Then the doctoral student placed the *Aim of the study*, which consists of three parts:

1. Verification of the applicability of the chronic unpredictable stress protocol for modeling depression in different strains of rodents based on the sucrose preference test.
2. Determination of 5-HT$_{1A}$ and 5-HT$_7$ receptors expression profile in different brain regions as the underling cause of heterodimerization between these serotonin receptors.
3. Determination of the possible involvement of 5-HT$_{1A}$ and 5-HT$_7$ receptors heterodimerization in the pathogenesis of depression using a model for the depressive-like behavior in mice

The *Materials* chapter is divided into two parts: one concerning theoretical research based on meta-analysis and the second one, an experimental part, in which the reagents used for biochemical analyzes are described in a very clear way in the form of seven tables.

The fourth, 13-page chapter is *Methods*, in which the PhD student describes the methods used in a very clear manner.

The obtained *Results* are described on 19 pages. The first part deals with the results obtained from the meta-analysis of chronic unpredictable stress protocols in rodents. The results were presented in the form of 7 forest plots and a figure presenting the research selection process. The next section contains the results of research on the role of 5HT$_{1A}$ and 5HT$_7$ receptors on the behavior of mice. The animals were tested with the tail suspension test after administration of the ligands of the tested receptors, 5-CT or LP-211. The author showed that stimulation of 5-HT$_{1A}$ and 5HT$_7$ leads to depressive phenotype in C57BL/6J mice and this effect is not associated with changes in locomotor activity.

Ms. Antoniuk also examined the mRNA expression level of the studied receptors by RT-PCR and protein levels using Western Blot in three brain regions: prefrontal cortex, hippocampus, and raphe nuclei on days P2, P12 and P90 of postnatal development. The obtained results are presented in three figures. Subsection 5.4. contains the results from chronic unpredictable mild stress procedure. Significant differences were found in the sucrose consumption in the sucrose preference test between the control or resilient and anhedonic groups. Moreover, an increase in the immobility time in the anhedonic group was demonstrated using the Forced swim test and a statistical decrease in body weight in both stressed groups (anhedonic and resilient).

Before discussing the results of the interaction between $5HT_{1A}$ and $5HT_7$ receptors, Ms. Antoniuk presented results from the colocalization of $5HT_{1A}$ and $5HT_7$ receptors in the prefrontal cortex, dentate gyrus of hippocampus and dorsal raphe nuclei. The results indicate that anhedonic animals present a decrease of $5HT_{1A}$-$5HT_7$ interactions in the medial prefrontal cortex, no change in the dorsal raphe nuclei and the resilient animals present an increase in the dentate gyrus of hippocampus. The results section concludes with a description of the results obtained for the tested receptor pair by RT-PCR and Western Blot methods in animals subjected to CUMS procedure in the brain regions mentioned above, where no significant changes were observed.

The discussion is written in a mature way with broad reference to the literature. The conclusions are adequate. However, it is a pity that the publication by Zurawek et al. 2019 was not discussed in the context of research on the $5HT_{1A}$ receptor and CMUS, as it showed in the CMS model on Wistar rats, that resilient animals have an increased level of the 5HT1A receptor. Moreover, this effect was observed only in parts of ca1, ca2 and ca3 of the dorsal hippocampus but not in the dentate gyrus of the hippocampus.

In Chapter 7., the PhD student briefly summarizes the findings presented in the thesis. The doctoral dissertation is crowned with an extensive bibliography of 220 literature items.

Ms. Svitlana Antoniuk's curriculum vitae and a list of three publications published by her are attached to the doctoral thesis.

The high editorial level of the dissertation should be emphasized. The work is very aesthetic, balanced with an elegant graphic design.

Critical notes

The results presented in the dissertation undoubtedly provide interesting information on the model of depression in rodents, the role of dimerization of serotonin receptors in the pathogenesis of depression, however, I have some questions or comments.

1. The methods section does not describe the animals used for the biochemical determination of receptor levels and mRNA expression at P2, P12, and P90.
2. Why is there such a different number of group individuals?
3. Fig. 13. Were locomotor activity measurements also performed for compound 5-CT?
4. Section 5.4.3 Proximity Ligation Assay. In good practice of GPCRs dimerization studies, it is necessary to demonstrate the lack of interactions of the studied receptors using genetic models with knockout of one of the receptors tested or using an siRNA method in order to silence the expression of one of the receptors. Have such experiments been performed?

5. On how many mice were PLA analyses performed?
6. How does the author explain the opposite changes in mRNA expression and protein levels of the studied receptors? (Figures 14 and 16).
7. Section 5.4.1 Results obtained for the resilient animals have not been discussed, nor the important issue of stress-resilience have been mentioned in the Introduction. What percentage of animals subjected to the chronic unpredictable mild stress procedure were resilient?
8. Section 5.4.3 shows the results of $5HT_{1A}$-$5HT_7$ interaction using PLA in human prefrontal cortex. I have not found information on this experiment before. Where were the preparations obtained from?

Final grade

In conclusion, PhD thesis by Ms. Antoniuk makes a significant contribution to the research on the role of GPCR dimerization in stress-related disorders. The relevance of these studies is strengthened by the fact that the PhD thesis has been supported by the European Union's Horizon 2020 research and innovation program under Marie Sklodowska-Curie grant agreement no 665735 (Bio4Med).

I hereby, declare that the reviewed Ph.D. thesis by **Svitlana Antoniuk** meets the criteria pursuant to art. 187 of Act of 20 July 2018r. on Act on Higher Education and Science (Dz.U. z 2021 r. poz. 478, 619, 1630) and request that the Scientific Council of the Nencki Institute of Experimental Biology, Polish Academy of Sciences accepts **Svitlana Antoniuk** for further stages of doctoral proceedings.

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Review of the PhD Thesis of Svitlana Antoniuk

Title: "INTERPLAY BETWEEN SEROTONIN 5-HT$_{1A}$ AND 5-HT$_7$ RECEPTORS IN STRESS-RELATED DISORDERS"

General Comments

The PhD Thesis by Svitlana Antoniuk investigates three important aspects of CNS function. First, serotonin (5-hydroxytryptamine, 5-HT) is one of the major central neurotransmitter systems involved in many neuropsychiatric and neurological disorders, including depression, schizophrenia, migraine, pain and movement disorder. The wide diversity of the activity of 5-HT provides rich opportunity for new discoveries, as shown by the ongoing research reported over many decades. Second, the Thesis investigates the effects of Chronic Unpredictable Stress (CUS) at behavioral and molecular levels in mice. The CUS model is one of the most instructive models of depression that is available and is highly informative for understanding the pathogenesis of mood disorders and their potential treatment. Third, the Thesis investigates the interaction between two important 5-HT receptors (5-HT$_{1A}$ and 5-HT7) that are known to be involved in regulation of mood and in influencing each other's signaling. Taken together, these elements mean that the theme of the thesis is likely to be of wide interest to researchers studying CNS activity.

The structure of the Thesis is clear and follows a standard format (Abstract, Introduction, Methods, Results, Discussion, References). Total length is 88 pages, including 220 bibliographic references. The work was conducted in two laboratories, one in Poland (Nencki Institute of Experimental Biology) and one in Germany (Hanover School of Medicine). Some of the work described in the Thesis has been published (Antoniuk et al., Neurosci Biobehav Rev 2019) and Svitlana Antoniuk has also made contributions to two further peer-reviewed publications.

Introduction

The Introduction starts by describing the prevalence of depression and its association with serotonergic mechanisms. There then follows a section describing the different 5-HT receptor subtypes but the largest proportion of the Introduction is focused on the cellular and molecular profiles of 5-HT$_{1A}$ and 5-HT7 receptors. Sections describing their signaling mechanisms and downstream effectors are supported by a range of bibliographic references. The accompanying illustrations (Fig. 2-4) are clear and particularly helpful.

A final section describes various models of depression and makes a useful distinction between those that were classically used, such as the olfactory bulbectomy procedure, in rodents and depression in humans. Indeed, the former has debatable relevance to the human condition whereas models based on chronic stress are much more pertinent to understanding depression in human subjects and model a wide variety of physiological dysfunctions, as are seen in a clinical context.

Some items that merit rethinking:

Page 13: The literature cited is extensive and generally appropriate but updated reviews of 5-HT receptor function deserved to be cited (e.g. Barnes et al. Pharmacol Rev 2021 PMID: 33370241, rather than Barnes et al 1999).

Page 14: It is odd that that no mention is made of Galpha-z protein coupling to 5-HT_{1A} receptors. In fact, this is important for control of HPA axis function by 5-HT_{1A} receptors, as well as influencing hippocampal serotonin responses (for example see Oleskevich et al 2005, PMID: 15931062).

Also, concerning G-protein coupling, it is important to note that 5-HT_{1A} receptors couple to different G-protein subtypes in different brain regions (see Mannoury la Cour et al Mol Pharmacol 2006, PMID: 16772521). This may underlie region-specific interaction / dimerization with 5-HT_7 receptors, as well as the activity of selective biased agonists (Newman-Tancredi et al. 2022, PMID: 34174274).

Page 15: there is mention of "several rare single-nucleotide polymorphisms (SNPs)" but the most obvious SNP affecting 5-HT_{1A} receptors is not mentioned, i.e., rs6295 which is functionally relevant and actually quite common (around 25% of Caucasian subjects). Importantly, it affects responses to antidepressant drugs (see Albert & Fiori 2014, PMID: 24180393 for review).

Page 24: The statement that 5-HT_{1A} receptors are "highly resistant to internalization when expressed alone" is too broad and is likely dependent on choice of cell line and/or experimental conditions. Indeed, an extensive study not cited in the Thesis was carried out by Kumar et al (2019; PMID: 30607822) showing that 5-HT_{1A} receptors do indeed undergo endocytosis when expressed alone. Moreover, studies carried out in the present Reviewer's laboratory show that receptor internalization can be used as a robust assay to characterize activation of 5-HT1A receptors by different agonists (Heusler et al EJP 2008, PMID: 18190908; Newman-Tancredi et al BJP 2009, PMID: 19154445).

Materials and Methods

The Materials and Methods sections are generally well structured. The lists of equipments and reagents look detailed. However, some aspects should be considered.

1. Page 39: there seems to be no description of the human brain experiments, except to say that "Brains were removed, post-fixed (mouse brain), or immersed directly (human brain) in 4% paraformaldehyde, cryoprotected in 30% (m/v) sucrose solution. Brain coronal sections (30 µm) were cut on a cryostat and processed for free-floating histochemistry."
   Important additional information should be given when using human tissues: what was the source of these human brains? Presumably they came from a biobank, but which one and where is it located? What are the characteristics of the subjects that gave their brains? Were they depressed patients or suicide victims or normal? What

was their age at the time of death and what ethical consent / approval was obtained for these experiments? This information is essential and will be necessary if the data is intended to be published in a peer-reviewed journal.

2. There doesn't seem to be any description of how anhedonic animals were distinguished from resilient animals. This is an important point because it underlies one of the major findings of the Thesis, i.e., that there is "a decrease in the number of 5-HT1AR/5-HT7R heterodimeric complexes in the stressed anhedonic mice in comparison to stressed control and stressed resilient animals" (as stated in the Abstract). The only methodological comment is on page 54: "Anhedonic animals were selected based on lower sucrose consumption in comparison to control and stress-resistant animals." This sentence is insufficient to understand what was done and, potentially, reproduce the experiment. Are the methods for selective resilient vs anhedonic mice the same as those published by the author in Krzystyniak et al (2019)? This might be the case, but it isn't stated: the procedure for differentiating these two populations of mice should be shown in the Methods section.

3. Concerning the drug tests, the Thesis describes the use of two agonists, 5-CT and LP-211. Concerning LP-211, previous publications show that it is active in vivo, so it is an understandable choice for these experiments. It would have been appropriate, however, to acknowledge that it has shown unusual insurmountable antagonist properties in vitro in HEK-293 cells, possibly due to stabilization of a Gs-coupled conformation of 5-HT_7 receptors (Atanes et al., Pharmacol Res Perspect. 2013, PMID: 25505568). Could this affect 5-HT_{1A} / 5-HT_7 interactions?
   Concerning 5-CT, what was the rationale for using this non-selective agonist? Possibly this was driven by cost (5-CT is cheap) but, on pharmacological criteria, it would have been far preferable to test a selective 5-HT_{1A} receptor agonist. There are various ones to choose from, including older 5-HT_{1A} receptor agonists such as repinotan, flesinoxan and tandospirone, as well as new highly selective biased agonists, such as NLX-112 and NLX-101. The use of 5-CT creates an ambiguity in the interpretation of the data because it activates both 5-HT_{1A} and 5-HT7 receptors, see further comments below.

Results & Discussion

The first part of the Results and subsequent Discussion concerns a meta-analysis of the effects of the chronic mild stress paradigm on different strains of rats and mice. This is an extensive review of the literature and clearly demonstrates that the chronic stress procedure produces deficits in sucrose preference across different labs and strains of rodents, although effect sizes and methodologies can vary from one study to another. The information is clearly presented and, as mentioned above, this forms the basis of a publication (Antoniuk et al., Neurosci Biobehav Rev 2019).

A limitation of this meta-analysis is the exclusion of all references that used the chronic mild stress paradigm but used sucrose consumption as a read-out, rather than sucrose

preference. This is discussed on page 66 where the main reason for not including those studies in the meta-analysis seems to be methodological variation between studies. It is somewhat debatable as to whether this represents a greater source of dispersion than the different procedures used for sucrose preference studies, notably in the duration and types of stress, as summarized on page 65. In the view of the present Reviewer, the relative merits of sucrose preference vs consumption remain under debate.

Page 49-64 and 71 onwards: These sections are at the heart of the novelty of the work in the Thesis, i.e., that (i) 5-HT_{1A} receptors are upregulated in the prefrontal cortex and hippocampus compared to the raphe nuclei during the brain development; and (ii) there is a decrease in the number of 5-HT_{1A}/5-HT_7 heterodimers in stressed anhedonic mice in comparison to unstressed control and stressed resilient mice. The first point, i.e. the changes in expression of 5-HT_{1A} receptors during development, has been previously investigated by various researchers and the present findings contribute additional information indicating that the relative expression of hippocampal sites decreases over time. The second point, i.e. that there are changes in heterodimer levels according to vulnerability to stress, constitutes a highly original discovery and has potentially important implications for the mechanism of action of antidepressant drugs. Additional findings of the Thesis are that there is a high level of expression of 5-HT_{1A} relative to 5-HT_7 in hippocampus but more balanced levels in PFC and Raphe. The study also found that the overall expression of 5-HT_{1A} and 5-HT_7 receptor mRNA and protein expression did not change upon exposure to chronic stress. The results describing these findings are clearly presented with clear histograms that are color-coded to facilitate readability. Normalization of the expression data to PFC or 5-HT_7 levels allows comparisons of expression levels between brain regions (e.g., Fig. 14) and between 5-HT_{1A} and 5-HT_7 receptors (e.g., Fig 15). The immunohistology of the different brain regions is similarly clearly presented and the images are of high quality, reinforcing the message of the quantified data. Overall, Chapter 5 is of high quality and merits recognition for a high standard of technical achievement. The results are then discussed in section 6.3.

The following comments should be noted:

- Page 49 and 66-67: both 5-CT and LP-211 increase the duration of immobility in the mouse tail suspension test (TST), with 5-CT showing a greater effect. The author concludes that “our results demonstrate that stimulation of 5-HT_{1A}R and 5-HT_7R in vivo leads to depressive phenotype in C57BL/6J mice”. However, this conclusion is flawed. Extensive literature evidence shows that activation of 5-HT_{1A} receptors has antidepressant-like effects in rodents in the TST as well as in the forced swim test. Here are some examples: Shang et al. 2021, PMID: 34101632; Sałaciak et al. 2020, PMID: 33103555; Miyata & Kamei 2004, PMID: 14628002. To determine whether both 5-HT_{1A} and 5-HT7 receptors are involved in the pro-depressive effects observed here, would necessitate experiments were a 5-HT_{1A} antagonist (such as WAY10063) or a 5-HT_7 antagonist (such as SB269970) are coadministered with 5-CT. In the absence of such experiments, the most probable interpretation is that the effects of 5-CT attributable to a predominant activation of 5-HT_7 receptors, not of 5-HT_{1A}. As mentioned above, it would have been preferable to use a selective 5-HT_{1A} receptor agonist, rather than 5-CT.

- Page 69: this section discusses the regulation of 5-HT_{1A} receptors in brain when investigated using PET labeling and highlights the fact that there is disparate data on whether depression is associated with increased or decreased 5-HT_{1A} receptor density. It would have been beneficial to note the fact that all the listed studies were carried out using antagonist radiotracers which label 5-HT_{1A} receptors in both G-protein coupled and G-protein uncoupled states. This can mask effects that are due to changes in G-protein coupling and highlights the need for agonist radiotracers, which specifically target G-protein-coupled receptors. One such radiotracer is [^{18}F]-F13640, which has been characterized in various species, including man, and also tested on post-mortem human brain tissue (for review see Newman-Tancredi et al 2022, PMID: 34174274).
- Page 72: there seems to be some confusion about the role of 5-HT_{1A} receptors on GABAergic interneurons as opposed to 5-HT_{1A} receptors expressed on pyramidal neurons. Activation of 5-HT_{1A} receptors on GABAergic interneurons does <u>not</u> inhibit glutamatergic signaling. In fact, activation of 5-HT_{1A} receptors expressed on GABAergic interneurons has an inhibitory effect on them, thus decreasing GABA release. In turn, this diminishes activation of GABA-A receptors on the pyramidal neurons, causing the latter's dis-inihibition (i.e., activation). Consequently, there is an increase in glutamate release from these neurons which activates post-synaptic AMPA receptors, thus mediating antidepressant activity. The picture below presents this sequence of effects (reproduced from Papp et al. BJP 2021, PMID: 34128229).

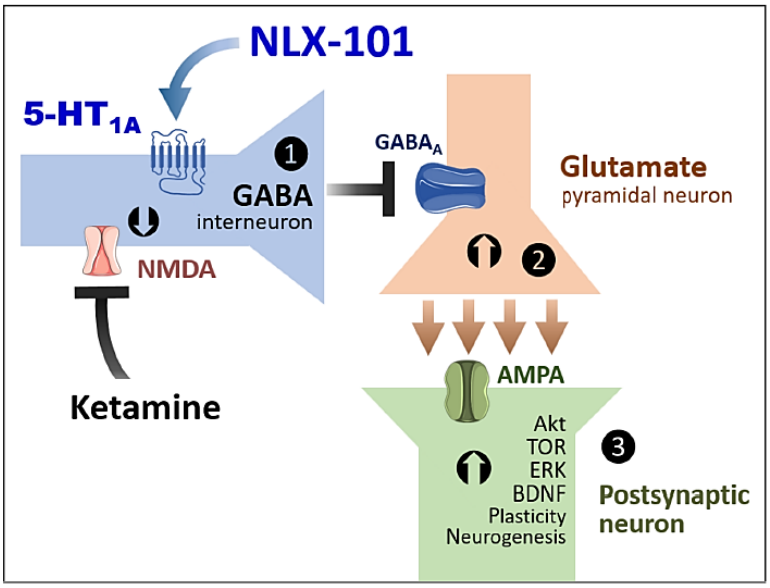

FIGURE 3 Converging cortical signalling mechanisms of ketamine and the 5-HT_{1A} receptor biased agonist, NLX-101. The analogous rapidly acting antidepressant (RAAD) effects of ketamine and NLX-101 may be mediated by converging signalling at cortical GABAergic interneurons (shown in blue). (1) Both the antagonism of NMDA receptors by ketamine and the activation of 5-HT_{1A} receptors by the biased agonist, NLX-101, inhibit GABA interneuron activity. (2) The reduction in $GABA_A$ receptor activity disinhibits glutamatergic pyramidal neurons (shown in orange), thus increasing glutamate release. (3) Glutamate activates postsynaptic AMPA receptors (shown in green) eliciting expression of neurotrophic factors, neuronal plasticity and neurogenesis, which mediate acute and long-term antidepressant activity. (), inhibitory effect; (), stimulatory effect. BDNF, brain-derived neurotrophic factor; TOR, target of rapamycin

- Page 74, minor comment: the conclusions state that there is an “increase in the number of 5-HT_{1A}R and 5-HT_7R heterodimeric complexes in the unstressed control and stressed resilient in comparison to stressed anhedonic mice.” It would be more accurate to say that there is a “decrease in the number of 5-HT1AR and 5-HT7R heterodimeric complexes in the stressed anhedonic mice in comparison to unstressed control and stressed resilient mice”.

A general comment about the Thesis, probably indicating that its findings are of potentially broad significance, is that it raises lots of interesting questions about extrapolations across species and to the clinic. Indeed, the findings of the study could be pertinent for understanding resistance to stress and the responses of pharmacotherapeutics that target 5-HT_{1A} and 5-HT_7 receptors, so it is tempting to consider what may be the implications for drug development. Moreover, at a basic science level, the different isoforms of 5-HT_7 receptors may influence how the present mouse data translates to other species: might there be disparities between mice / rats / humans? Is it possible to hypothesize what those might be? The fact that the work described in this Thesis raises numerous questions is likely a mark of its broad interest and it is to be hoped that the present line of research will be continued.

In any case, the results described in the Thesis make a valuable contribution to the field of serotonin research and shows that Svitlana Antoniuk has successfully carried out a technically demanding and ambitious project. She has shown that she has mastered a broad and complex range of technical investigation approaches and is able to present her results in a cogent and documented manner.

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