Dissertation thesis review – PharmDr. Chrysostomos Charalambous

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Thesis title: The role of ghrelin signalling in the neurobiological mechanisms of rewarding effects of cannabinoids and opioids

The author has written a thesis on 82 pages, out of which 66 belong to the thesis itself, using 179 cited references to scientific literature. The thesis has the usual structure with a theoretical part and experimental part, and is based on six original papers by the author.

The thesis focuses on the importance of ghrelin signaling for the neurotransmitter (esp. dopamine) response to addictive substances (opioids and cannabinoids) in the *nucleus accumbens* shell, which is considered a crucial impulse of the addiction process. The topic is of considerable importance, as the mechanisms of establishment and maintenance of addiction could be used in future addiction therapy or prevention. However, the findings are rather incremental than revolutionary, as the response to ghrelin receptor inhibition has been already described in multiple models of addiction, and the presented observations fit the same general pattern. There are some specific problems, issues and questions specified below.

Overall, the thesis fulfills the criteria for a doctoral dissertation, and I recommend it for thesis defense and awarding the author the title Ph.D..

Scientific issues. In Chapter 1, **Introduction to the problematic of addictive substances and addiction**, the sections 1.1 and 1.2 are dedicated to "psychoactive drugs" (usually it includes both illegal drugs and legal drugs of various classes). Judging from some statements ("The source of psychoactive drugs is primarily available from the official health system"; "Addiction is often of iatrogenic origin"; "Psychoactive drugs are often overused in the context of self-medication for pain, anxiety, etc.,"), the term "psychoactive drugs" is used here to designate *medical* psychoactive drugs specifically (such as painkillers, sedatives etc.). However, this is not clarified, which leads to confusion. Recreational drug use is not even mentioned in these sections at all, which is strange given the focus of the thesis on cannabinoids, where recreational use presumably dominates over prescriptional use or self-medication.

Practical impact of the findings and their relevance for further research directions is not sufficiently discussed.

Methodological issues. With statistics, some ANOVA results seem to compare only 2 groups at a time (e.g. 8.4). The correct way is to use ANOVA for all groups in a given experiment, and then to perform post-hoc tests to find specific group effects and/or specific timepoints where significance occurs. It appears that some type of post-hoc test was applied to determine specific timepoints where significance was reached, but it is not specified in the statistical methods.

Considering the behavioral methods, if the rats lived in groups of three per cage (7.1, P36), how did LABORAS distinguish individual rats if it used just mechanical vibrations? Were the measurements calibrated somehow, or at least validated by cross-checking the automated output with visual observations?

In the outputs, the parameters of Locomotion duration, Distance and Average speed are obviously highly correlated (which is natural, as they basically show the same thing), and therefore it would be sufficient to use just one measure of activity. Also, it would be interesting to know how the LABORAS system actually works and calculates its outputs, as the values of speed do not match the values of distance.

> Control rats have walked approximately 1 m per 20 min session (Fig 11D and Fig 12D), i.e. 1000 mm per 1200 seconds. This gives average speed of 0.83 mm/s. If the speed was calculated only for the duration of locomotion (panel A), it would be 1000 mm per approx. 12 seconds, which is about 83 mm/s. Neither number fits the values in Fig 11E and Fig 12E even in orders of magnitude.

The values of distance themselves (1 m per 20 min in the saline group) seem extremely low (in our experiments, although in a different setting, we had saline-treated rats walking 60 m per 20 min during the light phase). Such a low activity suggests either some serious calibration issue, or almost no locomotion in the rats (e.g. freezing due to stress). Both may affect the interpretation of the results.

Formal issues. The thesis has a standard structure and is of appropriate length. List of abbreviation, promised in the table of contents, is missing, and some abbreviations are not explained anywhere (e.g. CeA).

Descriptions of particular techniques used in the experiments (Chapter 5) would be better suited for the Methods section than the theoretical introduction. Models of addiction (such as self-administration paradigms) are not described in Chapter 5 despite its title.

Typographic errors are very rare, although they do occur (e.g. "theoritical part"; "oregenic" instead of "orexigenic"). However, the thesis seems to be poorly written in general and more attention to the text would be needed. Chapter titles not always fit well with the contents or main focus, some information is repeated needlessly etc. Some sentences are exactly repeated.

> P22: "The likelihood of non-standard acquisition of substitution drugs increases addiction, tighter dispensation control, lower availability of drugs, including affordability and underdosing while in treatment (non-compliance) (NMCDA 2019)."
> P23: "The likelihood of non-standard acquisition of substitution drugs increases addiction, tighter dispensing controls, lower availability of medicines, including affordability and underdosing in treatment (Mravcik et al. 2018)." (note different reference!)
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> P23: "Extensive knowledge of the complex neurobiological mechanisms of drug effects and their dependence mechanisms is essential for the development of effective treatment strategies, including the discovery of new drugs." (repeated 2x)

There are wording issues in some sentences, ranging from slightly unusual to outright awkward and compromising understandability. In some sentences, words appear to be missing. These issues occur for example in Czech abstract, chapter 2.3, the second paragraph of conclusions (P65) and elsewhere.

> P29: "CB1 antagonist in rats inhibited the orexigenic effect of centrally (intracerebroventricularly) ghrelin" – word "administered" is missing
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> P47: "Thus, the WIN55,212-2 + saline induced 2-AG decrease in the NACSh" contradicts the findings reported above and in Fig. 8. Probably should be "GABA" instead of 2-AG.

References to institutions should be clear and unambiguous.

> P31: "Institute of Chemistry and Technology (ICT)" is referred without further specifications, probably it is the same as "University of Chemistry and Technology Prague".

In the references, the use of "_____" instead of repeated author names is unusual, unnecessary and confusing.

Figures. Figures illustrate the most important principles and results. In Fig. 1, the colors of arrows in the schematic should be explained. Abbreviation "DO" instead of "DA" for dopamine in the legend.

Questions

Was anaesthesia of any kind used during intracerebral drug applications? Was the procedure accompanied by any signs of stress or seizures, which may occur in conscious animals?

Why 2-AG reacted differently to WIN55,212-2 (increase) and fentanyl (decrease)? Is it effect of drug type (cannabinoid vs opioid) or administration route (intracerebral vs systemic)? Why did it behave so differently from anandamide? What could be the relevance of these findings?

Why did JMV2959+fentanyl led to "undershoot" of anandamide deep below baseline? JMV alone had no measurable effect, so how would you explain this interaction?

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