REVIEWER #2:

We would like to express our thanks to the Reviewer for his/her contribution to improve the manuscript. Here, we fully considered his/her comments and criticisms. Please note that, in addition to highlighting the changes to the manuscript in the revised version, below, we underlined the texts of the manuscript that were either included or revised and highlighted them in green font. We used the strikeout format and red font to indicate texts that were deleted from the revised version.

Comments and Suggestions for Authors
The authors describe that they used label-free proteomics to identify deregulated proteins in the medial prefrontal cortex of male and female mice modeled to schizophrenia with a history of nicotine use during adolescence. Four biomarker candidates (Amph, Fkbp1a, Hspa1 and Eno2) were identified and had been discussed in article. They had appropriateness of study design; however, the results and should be replicated and validation by other methods. My major concern is the validation.

Label-free proteomics for quantitation is popular in the proteomics community and may be the most straightforward laboratory technique in the field. However, a drawback of label-free proteomics is that run conditions (e.g. temperature, experimenter, column condition) may differ between samples. In my opinion, the major issue is that in the absence of any validated data, such as immunoblot validation and biological replication, the authors cannot conclude anything about the role of proteins in the current study in the pathophysiology of schizophrenia with a history of nicotine use during adolescence. If possible, the authors should validate proteins they found, especially Amph, Fkbp1a, Hspa1 and Eno2, by immunoblot, which confirmatory analysis will improve the level of this study in a significant manner and give a better backing for the conclusions.

Response: We thank the reviewer for this comment. We agree with the reviewer in the sense that the validation of proteomic data by complementary techniques is useful in studies of differentially expressed proteins, however, it is not mandatory. Validation of protein abundance data obtained through mass spectrometry requires the use of a more refined, sensitive and robust technique. When considering label-free proteomics, this is only possible through experiments of MRM type (multiple reaction monitoring), which are also based on mass spectrometry, but which, due to the high cost, are often not feasible to implement. In this regard, articles recently published in IJMS that used similar methodological approaches to those used in the current study show proteomic data and ontological analysis without further validation of the implemented technique [e.g. Carrozziniet et al 2023. doi: 10.3390/ijms241311194 (https://www.mdpi.com/1422-0067/24/13/11194; Rochín-Hernández et al 2023. doi: 10.3390/ijms241612606 (https://www.mdpi.com/1422-0067/24/16/12606)]. The same occirs in other MDPI journals [e.g. Kudryashova et al 2023. doi: 10.3390/data8070119 (https://www.mdpi.com/2306-5729/8/7/119); Di Luca et al 2023. doi: 10.3390/ani13142263 (https://www.mdpi.com/2076-2615/13/14/2263).

Mass spectrometry is advantageous when compared to conventional antibody-based methods such as Western Blotting, as the former offers greater specificity,

reproducibility, linearity, and quantitation limits below the fmol range (Jayasena et al 2016. doi: 10.1038/srep35391). Techniques based on immunoassays are more easily automated and require less training but are more susceptible to interference and have a smaller detection range when compared to mass spectrometry. Label-free quantitative proteomics as a rule often have lower quantitation limits than immunoassays and therefore are useful for measuring much lower concentrations of analytes (https://www.medlabmag.com/article/1290). In addition, the Hi-3 quantification method used by our group is very well described (Silva et al 2006. doi: 10.1074/mcp.M500230-MCP200) and is based on the recognition of the 3 most abundant peptides of each protein to finally obtain the mean protein abundance. When the protein does not contain all 3 unique peptides, Progenesis QI for proteomics performs protein quantification in two steps, the first step being performed only with the unique peptides, which provides an estimate of the protein abundance. The second step is performed using peptides that can be shared by other proteins, determining the abundance, and distributing the abundance value in the proportion of the proteins that share the non-unique peptides.

Regarding the Reviewer's concern about the stability of run conditions:

A) Factors such as temperature, experimenter, column condition and other experimental parameters used in the current study were consistent with a robust high-level protein quantification experiment. The analytical column and the sample manager temperatures were continuously monitored by the equipment, which warns of small temperature variations (±1 ºC). These temperatures were maintained at 35°C and 9°C, respectively, during the 96 chromatographic runs, totaling 7200 minutes of running without significant variation in temperature parameters.
B) The chromatographic column condition between runs was monitored by injecting MassPREP™ ADH digestion standard (100 fmol) every 12 runs of samples, when both chromatographic complexity (number of resolved peaks), resolution of chromatographic peaks, retention and signal intensity. All the conditions of the protein standard mix respected the parameters evaluated throughout the experiment.
C) To minimize the "experimenter" effect, pools of biological samples were made, analyzing 4 biological samples per group and 3 technical replicates of each sample to guarantee the robustness of the study and to increase confidence in the data presented (please refer to Methods, page 6, lines 200-205). Other monitored experimental parameters (mass spectrometer calibration, signal intensity normalization, run reproducibility, trypsin enzymatic efficiency, mass error) were also maintained within acceptable levels for this type of study during all runs.

Considering the Reviewer's concern, which might be shared with the readers with expertise in mass spectrometry, we modified the text of the manuscript to briefly describe the approaches adopted to minimize methodological variations throughout the experiment. The need for future studies to confirm our results and to further investigate the mechanisms that underlie schizophrenia+nicotine misuse comorbidity was also added to the Discussion section.

Highlighted version (Method, page 7, line 257)

To minimize methodological variations that might impact the results, during all runs, the analytical column and the sample manager temperatures were monitored and maintained constant. The chromatographic column condition was also monitored every 12 sample runs.

Highlighted version (Discussion, page 26, line 831)
The methodological approach used in the current study allowed the identification of potential pathological markers of the combined phencyclidine and nicotine exposures during adolescence.

Highlighted version (Discussion, page 26, line 842)
(…) raise the possibility that SCHZ candidates reported in previous studies are in fact biomarker candidates for SCHZ+nicotine misuse comorbidity. Accordingly, future studies to confirm the obtained results by using suitable cellular models and molecular tools aimed at clarifying the mechanisms of schizophrenia+nicotine misuse are warranted.

Highlighted version (Discussion, page 27, line 858)
In this sense, the current explorative study has the potential to direct future studies aiming to identify mechanisms of nicotine interference in the early course of SCHZ, and possibly, to create more efficient treatments tailored for early intervention

Line 25: should add "(Amph)" after Amphiphysin.

Response: It was added to the revised version

The Introduction should be concise.

Response: We have taken efforts to reduce the introduction extension, but please note that new information was also added, as suggested by Reviewer 1.

Highlighted version (Introduction, page 1, line 35)
The available pharmacological approaches to treat this disorder are largely based on the manipulation of neurotransmitter systems activities, ~~with a major focus on the dopaminergic one but also with interference in serotonergic and muscarinic, as well as GABAergic and glutamatergic systems [3,4]. The~~ and their partial efficacy ~~of these pharmacological approaches~~ has fueled the development and acceptance of the main hypotheses of SCHZ, ~~which revolve around the deregulation of neurotransmitter systems function~~ [3,4]. Corroborating these hypotheses, alterations in ~~the aforementioned~~ several neurotransmitter systems have been identified in SCHZ patients [5] and in animal models [6].

Highlighted version (Introduction, page 2, line 65)
~~The cholinergic system is the primary site of action of nicotine. Its~~ Nicotine binding to nicotinic cholinergic receptors (nAChRs) not only has a profound impact on ~~this~~ the cholinergic system but also, indirectly, modifies the functioning of other neurotransmitter systems [26,27], several of those involved in the SCHZ pathophysiology [27,28]. ~~Accordingly, the central nicotinic cholinergic system has been suggested to mediate the putative beneficial (and detrimental) effects of nicotine on SCHZ. Despite that, so far, no nAChR ligand has been approved by~~

~~regulatory agencies to treat SCHZ [29,30].~~ However, so far, the mechanisms that underlie nicotine effects on SCHZ symptomatology are not established. This ~~failure~~ could be due to the involvement of pathways other than those associated with the classic neurotransmitter systems.

Highlighted version (Introduction, page 3, line 86)

(...) from 59 to 78% in patients with first-episode psychosis when compared with healthy matched controls [42,43]. ~~These last numbers are compatible with the disturbing odds of 6 for tobacco consumption in first-episode psychosis [42].~~ These findings indicate that the association between SCHZ and nicotine misuse is established early.

Highlighted version (Introduction, page 3, line 99)

~~Finally, the mPFC communicates with several brain regions involved in both mental disorders, including the nucleus accumbens [51], amygdala [46], and hippocampus [52].~~

Highlighted version (Introduction, page 4, line 129)

~~Phencyclidine administered on a daily basis guarantees the development of deficits associated with SCHZ [61]. As for nicotine, exposure was restricted to a short period so that the impact of nicotine history on the proteome of mice modeled to SCHZ could be evaluated.~~ We hypothesized that the analysis of the mPFC would lead to the identification of key pathological markers of the combined insult, which would correspond to exclusively deregulated proteins in mice modeled to SCHZ and nicotine misuse comorbidity.