Dear Dr. Lehman,

Thank you very much for the comments regarding my manuscript on adolescent stress and hypothalamic gene expression (JNE-25-0038-OAr). We have modified the manuscript according to the reviewers’ comments. Here is the outline of these changes as well as replies to reviewers’ comments, in bullet points under each original comment. All changes are highlighted in the updated manuscript and referenced by line number in these comments.

**Referee: 1**

**Comments to the Author**

**JNE-25-0038-OAr REVIEW**

**This paper uses “global transcriptomic approach” to identify altered gene expression in the hypothalamus of male golden hamsters (M. auratus) in response to chronic social stress. This experiment therefore aims to identify novel transcripts regulated by this chronic stress model. This is a small and defined experiment, that does not describe a novel phenomenon, and requires some additional validation in order to justify that gene expression changes are truly in the brain regions investigated (achievable through interrogation of existing data).**

**MAIN COMMENTS:**

**The title, abstract and introduction neglect to mention what species of hamster this paper describes. The first mention of what kind of hamster the authors work with is currently in the methods. This is an oversight since many distinct species of hamster, with divergeant physiology, phenotype and regulation are used as model species. The type of hamster should be named in the title, introduction and abstract, with latin name, to avoid any ambiguous interpretation.**

* Thank you, we have clarified the species in the places provided (LINES 1, 19, 60)

**Similarly, the authors should make sure reference to published literature specifies what species of hamster the reference is concerned with.**

* We have clarified within the introduction that the species being discussed within cited references is the Golden hamster (LINES 61, 65, 72, 99).

**In methods the authors explain why they only used male hamsters, it seems that they are investigating a phenomenon that only occurs in male hamsters, and therefore should be justified earlier on in the manuscript.**

* We have added clarification and additional literature reference to (LINE 72-73) to note that the effects discussed are more prominent in males than females.

**Introduction:**

**- “in many humans” – under what circumstances? Can the authors be more specific about what they mean here? Why not in all humans?**

* We have added some additional context for contributing factors of this effect to (LINES 55-58). While the fact that this effect does not occur universally is interesting – and a major reason to study a diversity of animal models to understand differences in underlying mechanisms – we did not include a paragraph on this because we did not want to get too off topic. We did publish a review last year (Moran and Delville, 2024) that begins to interrogate such a question, however.

**- different behavioural outcomes” – What is the Wommack reference relating to here? Please specify these outcomes and how they differ.**

* Thank you for highlighting that the introduction of this paragraph might be unclear – the whole paragraph discusses the behavioral changes, and we have added some clarification to (LINES 73-75) to clarify this.

**Methods**

**- While the time of day was indicated for the timing of experiments, it would be more useful to state this relative to the timing of lights on (Zeitgeber time).**

* We have further clarified that all experimental procedures occurred during the dark/active cycle of the animals and used a ZT reference (LINES 123-124).

**- “Metabolic Metrics / Metabolic Measures” are not described in the methods (only how they were statistically compared), please add this.**

* We have added all the metabolic metrics recorded and used for comparison (LINES 206-207).

**- It would be useful to have a little more detail on how tissue punches were collected. Presumably from already frozen brains, and then were the brains thawed and sectioned? How thick was the section that the punch was taken from / how far back from the stated coordinates were the tissue punches collected from? This is important as these brain regions are distinct and close together.**

* We have added a number of clarifying details to the tissue punching procedure (LINES 165-166, 169-170). We have also added supplementary figure 1 and LINES 238-241 to support distinction of sampling.

**- Figure 1 in the methods is a welcome explanation of the experimental design, however panels A and B are quite close together so it looks like the different brain regions were collected from hamsters at different ages. Please separate these panels a bit more, and/or change alignment of the coronal sections to the age of the hamsters.**

* Thank you for allowing us to clarify this figure, a new version has been submitted.

**RESULTS**

**Aside from the “region incongruence” (table 3), did the authors do any validation of the brain regions they collected in terms of expected high/low gene expression markers for each region? How confident are they in their dissection? This is likely an important control to validate this work.**

* Regions were validated by separation in principle component analysis (newly added Supplemental Figure 1) and presence of marker genes that are commonly known to be in these regions (hcrt in the LH, npvf in the DMH, and npy in the ARC) (de Lecea et al., 1998; Jhamandas and Mactavish, 2002; Tatemoto et al., 1982). This has been added to (LINES 238-241).

**DISCUSSION**

**The discussion is largely unproblematic although a little lengthy. However, following on from my previous comment - one of the identified transcripts discussed is GPR50 – is it likely that this could be an artefact of dissection, as this is mainly thought to be expressed in the ependymal layer (tanycytes) of the third ventricle, how much expression would you usually expect to see in the LH or DMH? Do the cited references describe effects in these regions? Perhaps this is the reason ARC sample expression was "not as expected”.**

* While the lack of GPR50 expression in the ARC could be an artifact (we have added this note on LINE), expression of GPR50 in the DMH and LH has been previously reported and discussed in rodents and humans (Khan et al., 2016; Sidibe et al., 2010), as is cited in the manuscript. However, the effects reported in our manuscript in the DMH could be related to effects in the 3rd ventricle due to their proximity. We have added this note in (LINE 396 and 503-509).

**“overall the outcome supported our initial hypothesis” – please restate that here. Or in fact state for the first time in the introduction, as this is not currently the case.**

* We now restate our hypotheses (from LINES 101-106) in the conclusion – “that a variety of genes and functional genetic modules related to metabolic processes and development were altered by stress exposure” (LINES 510-512) for clarity.

**Since the analysis here way only concerning the hypothalamus, the authors should keep their conclusions to this rather than “multiple systems”.**

* We have specified that our analysis involved only “hypothalamus-related systems” (LINE 513).

**Referee: 2**

**Comments to the Author**

**This is a well-designed study looking at the effects of chronic social stress on transcription in hypothalamic nuclei.**

**Overall, while the results seem quite descriptive, this can provide an interesting resource that could supplement existing data from other species.**

**The results seem to have been analysed well, using a fairly standard pipeline; I have a few minor comments:**

**- In Figure 2, there seems to be a larger variability in the stressed groups compared to controls. It almost looks like the stressed groups are bimodal, particularly for fat mass and possibly weight. Could the authors comment on that?**

* It is a keen observation that there is variability in how hamsters metabolically respond to stress. In past publications from the lab, we have investigated if there is any correlation between these metrics and a variety of other factors measured through previous experiments, such as metabolic metrics (Moran et al., 2021, *Hormones and Behavior*), food conditioned place preference behaviors (Moran et al., 2025, *Behavioural Brain Research*) or orexin innervation in a variety of brain regions (Moran et al., 2025, *Journal of Neuroendocrinology*). Very rarely were there any correlations. We have added this clarification to the methods section (LINES 150-156). However, we have also added additional analysis between these stress behaviors and gene expression of the most differentially expressed genes (LINES 210-217, 303-309) and Supplementary figure 3, and a brief addition to the discussion (LINES 492-502).

**- Caption of Figure 2A should not say "significantly different measures", given not all of the measures are significantly different.**

* We have removed this wording to make the caption more accurate.

**- In Figure 3 it is unclear what the four vertical lines represent. Usually, two lines are used to indicate the upper and lower log2 fold change threshold, but it does not seem to be the case here. Furthermore, a threshold for log2 fold change of >0.2 (i.e. a 1.15 fold change) seems quite low, and I would question the biological relevance of such small changes. Could the authors comment on this? I agree that thresholds are purely arbitrary, but this seems to be much lower than what is typically used (e.g. 1.5 fold change, so a log2 fold change of 0.59 or even 2 fold change, so a log2 fold change of 1).**

* Dashed vertical lines at |0.75| and |1.5| are included to illustrate numbers of genes at different ranges of change, and this has been added to the figure legend.
* We agree that these thresholds are arbitrary, and that a 15% change in expression may not necessarily be biologically relevant. However, prior published work has used 15% as a cutoff (e.g. Lee et al., 2022, *Hormones and Behavior*). Additionally, the very consistent, reproducible magnitude of the body weight changes that occur in the stressed hamster phenotype are also usually at the scale of 10-15%, so this scale can be very biologically meaningful. That said, we are typically only discussing specific genes when they have a much larger fold change or a very low p-values (i.e. very little overlap between groups in expression) or both.

**- As a very minor point, I find it a bit odd that the volcano plots show fold changes using stress as the reference level instead of control. This is not wrong, of course, but I would expect a positive fold change to indicate upregulation in the stressed group compared to the control group.**

* In hindsight, we agree that using the control group as the reference point (causing the stressed group’s increased expression showing as negative) can be confusing. Throughout the manuscript and figures we make sure to clarify the meaning of the directionality of results for this reason. We will take this into consideration in future work.

**- Can the authors describe more precisely how the "top genes" in the table of Figure 3 were selected? I have no particular problem with the authors selecting the genes based on (presumably) biological relevance amongst those with low p-values and/or high fold changes, as it is difficult to present these data in a completely unbiased way when so many genes are changing. However, it does not seem like all genes above/below a specific threshold are listed, so maybe instead of "top genes" these should be listed as "selected genes", otherwise a more precise criterion for choosing them should be described.**

* Top genes were selected by filtering the highest fold change while having an eFDR < 0.05. (this is now included in the figure legends of Figure 3 and Table 1). We apologize for the mistake here causing the confusion. The figure embedded in the manuscript was from an earlier draft of the paper, while the high quality figure 3 submitted as a PDF is an updated version that adheres to the selection criteria in the “Top genes” column as described in the legend now.

**- Is there any relationship between the metabolic measures in Figure 2 and gene expression? For example, the authors might try and map those parameters onto a PCA projection of the data points and see if some specific pattern emerges. Furthermore, the authors might wish to comment on the relevance of the transcriptomic changes seen in their study for generating those metabolic effects.**

* We hope we addressed the first part of this point in response to the reviewer’s first comment. That said, we did discuss a number of potentially biologically meaningful gene changes in our discussion, including discussion of orexin (*hcrt*), Neuropeptide W (*Npw*), Neuropeptide VF precursor (*Npvf*), melanocortin 3 receptor (*Mc3r*), among others, and functional modules related to immune function, all of which have potential metabolic roles and consequences.

**A discussion of the study limitations, e.g. but not limited to the fact that samples contain mixed neuronal populations, would be beneficial.**

* We have added a section of the limitations of sampling from such a heterogeneous region as well as the limits of a bulk RNA sequencing method to (LINES 503-509)

**- I did not see a data availability statement. To enhance transparency and reproducibility, I strongly recommend that the authors deposit both the raw and processed data in a public repository. Additionally, sharing the analysis pipeline would allow others to validate and build upon this work.**

* We apologize for the confusion! We uploaded all our raw data to GEO accession prior to submission and the analysis pipeline in a Github repository and stated our plan to release it upon publication, but perhaps this statement got lost. This is still our intention in pursuit of transparency and reproducibility.

**Referee: 3**

**Comments to the Author**

**This study used a hamster model and RNA Tag-sequencing to identify gene expression changes in the hypothalamus following chronic social defeat in adolescence. This lab has a considerable body of research on the effects of chronic social defeat in adolescent hamsters and the current study was designed to identify genes associated with several of the known behavioral and physiological outcomes in this model. They found that stressed adolescents showed changes in the expression patterns of a wide variety of hypothalamic genes including those regulating metabolic processes, myelination, neurodevelopment. The study is straightforward, and the manuscript is well written. However, I do not have expertise in RNA sequencing, and I will leave it to other reviewers to comment on the RNA Tag-sequencing methods. The authors do a commendable job in the Discussion section describing relevant gene expression changes and placing in the findings in a functional context. My comments below are minor.**

**1. Page 7. Explain whether new fighter males were used for each social defeat exposure.**

* Adolescents were rotated through seven adult males, such that they did meet the same male more than twice and did not form a stable relationship with any of them. This has been added to (LINES 139-141)

**2. Page 7. The researchers quantified the amount of aggression received and the amount of submissive behavior exhibited by subjects. I suggest the authors report these data. It would be helpful to document how much aggression occurs during these encounters. There is a substantial amount of data available in the literature about how much aggression adult hamsters receive during social defeat exposure, but much less data available for adolescents.**

* We appreciate the interest and have added: “On average, Stressed subjects received 1-2 attacks per day, displayed 1-3 tail up behaviors per day, and 0–1 of the other recorded social behaviors were scored.” This has been added to (LINES 150-156). We have reported similar numbers in adolescents in (Moran et al., 2025, *Journal of Neuroendocrinology*).

**3. Page 7. Inter-rater reliability for quantification of aggressive behavior should be reported.**

* There was only one observer during aggressive behavior exposure. In past publications from the lab, we have investigated if there is any correlation between these metrics and a variety of other factors measured through previous experiments, such as metabolic metrics (Moran et al., 2021, *Hormones and Behavior*), food conditioned place preference behaviors (Moran et al., 2025, *Behavioural Brain Research*) or orexin innervation in a variety of brain regions (Moran et al., 2025, *Journal of Neuroendocrinology*). Very rarely were there any correlations. We have added this clarification to the methods section (LINES 150-156). However, we have also added additional analysis between these stress behaviors and gene expression of the most differentially expressed genes (LINES 210-217, 303-309) and Supplementary figure 3, and a brief addition to the discussion (LINES 492-502).

**4. Page 8. Samples are often pooled in RNA-seq studies. Explain whether samples were pooled across individuals or across hemispheres for this study.**

* Bilateral punches were taken from two sections for each region per animal and pooled for sampling. This is now explained in (LINES 169-170).

**5. Page 13. I suggest labeling Figure 2C white adipose tissue in the caption or on the data plot.**

* The figure caption has been changed to more accurately describe the fat collected as “white adipose tissue pads collected.”

**6. Pag 23. Firing mechanisms is confusing. Please explain.**

* We have added more context to what we meant by “firing mechanisms” in (LINES 348-350).

**Referee: 4**

**Comments to the Author**

**This is a largely discovery-based approach to begin to identify genes that might be involved in the effects of developmental social stress on metabolic and behavioral endpoints. Hamsters are a particularly interesting model animal to use in these studies because they have been widely used in studies of social stress during development and in adulthood. The study is well-controlled and the methodology is for the most part appropriate. Addressing the following concerns would greatly strengthen the manuscript:**

**1. The abstract needs to be more explicit in terms of what the purpose of the study was.**

* Thank you for the suggestion, we have added more detail to the abstract clarifying the purpose and goals of the study (LINES 22-26).

**2. Need to expressly justify/explain the brain areas examined. It is not clear why these areas were chosen as opposed to others that might have been just as interesting.**

* We have expanded on our introduction paragraph that explained why these specific ROIs were chosen for analysis in (LINES 94-100).

**3. A definite weakness is that behavior of both resident aggressors and subjects was scored concurrently live by only one observer. This does not appear to be a very reliable or rigorous way to obtain behavioral data. Furthermore, it is not clear if or how these data were used. Perhaps the authors could elaborate and clarify? Was this done only to judge whether the subjects were defeated or not? Was it done for every bout? Were the data analyzed? Could there be any correlations among differentially expressed genes and behavior?**

* In past publications from the lab, we have investigated if there is any correlation between these metrics and a variety of other factors measured through previous experiments, such as metabolic metrics (Moran et al., 2021, *Hormones and Behavior*), food conditioned place preference behaviors (Moran et al., 2025, *Behavioural Brain Research*) or orexin innervation in a variety of brain regions (Moran et al., 2025, *Journal of Neuroendocrinology*). Very rarely were there any correlations. We have added this clarification to the methods section (LINES 150-156). However, we have also added additional analysis between these stress behaviors and gene expression of the most differentially expressed genes (LINES 210-217, 303-309) and Supplementary figure 3, and a brief addition to the discussion (LINES 492-502).

**4. Methods do not specify the sex of the subjects; please add and describe how culling of litters was done in terms of sex of animals, as well**

* We have added additional clarification that the experiment uses males subjects and why (LINE 72, 113) on top of our existing justification (LINES 129-132).

**5. Tag-seq is reliable in well-annotated genomes. The authors might consider expressly stating whether the hamster genome is considered well-annotated (with appropriate references).**

* The newer BCM\_Maur\_2.0 genome has 21,616 annotated protein-coding genes and 10,459 annotated noncoding genes compared to 20,495 protein-coding genes and 4,168 noncoding genes in the MesAur1.0 genome, and over 97% of reads are mapped. While not as thorough as mouse or rat genomes, the BCM\_Maur\_2.0 genome is highly mapped and annotated. This has been added to the methods (LINES 224-225).

**6. Terminology: The authors use the more careful description “differentially expressed” genes in some places but use “differentially regulated” in others. The latter suggests a deeper/more mechanistic level of analysis, which was not done here. Perhaps it would be better to stick with differential expression. Relatedly, in the Discussion the authors often appear to discuss changes in peptides or receptors instead of changes in gene expression. The latter does not necessarily mean changes in the former given how much posttranslational processing of peptides and receptors occurs.**

* We definitely understand the need for clarity regarding terminology, thank you for bringing this to our attention. We have gone through the manuscript and altered our wording in many locations to refer to increased/decreased expression of gene transcripts instead of up- or downregulation. We have also clarified in multiple locations that we are referring to gene expression changes, not necessarily peptide or receptor changes.

**7. The significance threshold is confusing. I’m not sure what “15% of change in the absolute values of log 2-fold change at the eFDR of 5%” means. This should be described more clearly and justified explicitly. The authors have described and supplied references for some of the analyses but not others. Try to explicitly support/describe the analyses as much as possible.**

* Thank you for allowing us to clarify this segment. It now reads: “The significance threshold for differentially expressed genes (DEGs) was set as a log 2-fold change of 1.2 (a 15% difference) with an eFDR of 5% (p < 0.05).” (LINE 235).

**8. In terms of the body fat measures, there was a trend (with a strong effect size) for a higher total body fat in stressed hamsters. Were there any separate measurements of fat pads or were they only pooled? It might be possible that there would be significant differences if a single fat pad type (particularly mesenteric) was compared between the two groups.**

* We decided to only present pooled fat pad weights here, as it was not the major focus of the paper. However, previous research has explored differences in separate fat pads individually (Moran et al., 2021, *Hormones and Behavior*). The present data reported are included as they support previous findings in this model.

**9. In the Discussion page 28, the second sentence in the paragraph beginning “Gene expression…” doesn’t make sense. “While others…” needs to have the other clause.**

* Thank you for bringing this error to our attention. This sentence now reads: “While others have found that adolescent stress in rats resulted in reduced Pomc and Cartpt expression in the ARC alongside reduction in body weight (Krolick et al., 2022), we observed no differences in expression of the classic appetite-related ARC systems such as NPY/AgRP or POMC/CART.” (LINES 460-463)

**10. The final paragraph of the Discussion should restate the hypotheses, if any, and describe how they were supported by the data. Don’t just say that the data supported the initial hypotheses. In fact, the only explicit hypothesis that was stated in the Introduction dealt with changes in expression of feeding-related transcripts. There were some predictions about other pathways or modules of genes that might be differentially expressed, but this was not detailed. The Discussion clearly goes way beyond this, reflecting that this is largely a discovery-based project. This should be wrapped up more appropriately in the last paragraph emphasizing what the authors think that the impact of this work will be.**

* We now restate our hypotheses (from LINES 101-106) in the conclusion – “that a variety of genes and functional genetic modules related to metabolic processes and development were altered by stress exposure” (LINES 510-512) for clarity. We have expanded our conclusion to discuss more potential use of the present data as well (LINES 515-518).

**Comments from the Senior Editor:**

**Your manuscript has now been reviewed by four expert referees. While each reviewer was supportive of publication, they highlighted some issues that require attention. I have also read your manuscript carefully and largely agree with their comments. In addition, while reviewing your manuscript I noted some other minor issues that should be addressed.**

**1. Provide information about how the metabolic data was collected in the methods.**

* Starting two days before the stress and control procedures, hamsters were weighed and had all food removed from their home cages and weighed, then “topped off” to 80g. Weighing always occurred prior to stress or control procedures. This process was repeated every two days, until the end of the stress period. Similar to other studies (Foster et al., 2006; Solomon et al., 2007), we analyzed food intake as cumulative food eaten over time. This has been added to (LINES 124-129).

**2. For the results of the metabolic metrics, when presenting the data in the text, provide the units e.g. grams (g will suffice).**

* Thank you for letting us clarify, these units have been added to the text (LINES 266-270).

**3. Figure 3: Can you define what the double up or double down arrows indicate in the figure legend. Presumably up- or down-regulated genes, but I am unclear what, if anything, is the significance of the double arrow?**

* Yes, ↑↑ indicates upregulated, ↓↓ indicates downregulated. There is no significance to the double arrow other than clarity – this has been explicitly added to the figure legend