nature portfolio

Peer Review File

Hypothalamic SLC7A14 accounts for aging-reduced lipolysis in white adipose tissue of male mice

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

What are the noteworthy results?

The study evaluated the mechanisms of regulation of lipolysis during ageing. Authors suggest that during ageing, the levels of POMC SLC7A14 are reduced leading to the activation of mTORC. This generates a sympathetic signal that reduces the expression of ASBT in enterocytes, thus, reducing reabsorption of TCDCA. Authors suggest that TCDCA acts through TGR5 in WAT to induce lipolysis. Despite the undisputed importance of the finding, there are several gaps that should be filled in before the whole story is ready.

Will the work be of significance to the field and related fields? How does it compare to the established literature? If the work is not original, please provide relevant references.

It is original, and it is important to the field.

Does the work support the conclusions and claims, or is additional evidence needed?

There are a considerable number of missing pieces. There is a need for quite a lot of additional evidence.

Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision?

Yes, as follows:

The characterization of SLC7A14 expression and distribution in the brain, and particularly in the hypothalamus was very superficial. Authors should determine cell specific expression of SLC7A14 using single-cell RNA seq. This is particularly important because it is unclear whether SLC7A14 is expressed in AgRP and POMC neurons in WT mice.

Out of the cells expressing SLC7A14 in the hypothalamus how many are POMC?

There is a tremendous gap of information between the effect of POMC SLC7A14 on TCDCA and the capacity of TCDCA to control lipolysis. I could not find any prior study that explored

such mechanism. Considering that the central issue of the present study is, how the hypothalamus of ageing mice control lipolysis, authors should go deep into the mechanism involved in TCDCA-dependent regulation of HSL.

Since authors propose that TCDCA acts through TGR5 to control lipolysis in WAT, why it is not acting through BAT TGR5 to stimulate BAT thermogenesis, a well know mechanism. What provides this tissue specificity?

Authors suggest that the regulation of ASBT by POMC stimulus could be mediated by PPARa, but this hasn't been explored in depth.

Is the methodology sound? Does the work meet the expected standards in your field?

There are some experiments that are not convincing and additional evidence should be provided

Authors should use retrograde tracers to provide undisputed evidence for the effect of POMC SLC7A14 on gut sympathetic control of ASBT.

Exploring the POMC signaling mechanisms involved in the response to SLC7A14 using immunoblot of hypothalamic cells is not appropriate in the context of such a multi organic, systemic phenomenon.

Is there enough detail provided in the methods for the work to be reproduced?

Yes.

Additional suggestions

The introduction is far too long. It contains a number of basic definitions that should be removed. Authors should provide at most two or three paragraphs of background strictly related to the central question, and then a final paragraph for raising the hypothesis and objectives of the study.

Reviewer #2 (Remarks to the Author):

Jiang et al. describe an interesting study on the involvement of SLC7A14 on the onset of age-related obesity. SLC7A14 belongs to a membrane transporter family, hence it is expected to play a transport function in cell (lysosomal) membrane. However, the author study is limited to a description of relationships among SLC7A14 expression/knocking out and regulation of obesity insurgence in mice through a pathway involving an intestinal TCDCA transporter. The work is well conducted on the experimental point of view but has two major flaws: i) no insights into the function of SLC7A14 are provided and correlated to the described role; ii) no molecular description of the mechanism by which a central nervous system district acts on intestinal absorption of a metabolite, as the same authors state at the end of discussion. At least one of the two issues should be dealt with and elucidated. Then, the manuscript should be changed in its structure, accordingly.

Specific comments:

Introduction must be rewritten trying to describe what is known around the subject (SLC7A14) and how it is connected with the other players (mTOR ect.) arriving to the final hypothesis. Major flows of introduction are listed below.

1. Only mTORC1 is described but not mTORC2 that, after, appear to be involved in SLC7A14 action.

2. Lines 66-70 can be removed since are useless for readers in the frame of the manuscript.

3. Line 75: TSC1 appears after description of TSC (explain what TSC1 is for non-expert readers).

4. Lines 78-79 seems in contrast with 75-76. Rephrase

5. Lines 85-88: the sentences seem to not be connected to the following one "solute carrier....". Which is the connection between SLC7A14 and the metabolites that can mediate the effects of the CNS on lipolysis? GABA/arginine may be involved?

6. Lines 95-98 are again not well connected. Which is the message?

7. From line 99 mTORC2 appears which had not been mentioned before but now may be the target of the subject of the manuscript.

8. Lines 101-104 put forward a well-defined hypothesis that is difficult to derive from the previous sentences and uncertainties.

Results.

1. Line 113 "highly expressed" is not appropriate since there is not a control of "normal" expression. It should be stated as example: "expression in HPO and HY is higher than expression in ...".

2. Legend of fig S2b is not clear: what - 22M means? Both 8W and 22M old mice? May be there is an error in the indication.

3. The same problem is present in Fig. 1 legend.

4. Line 131 please report the extended name of enzyme (Hormone sensitive lipase?)

5. Line 202, the sentence lacks something or the comma must be eliminated.

6. Lines 221-222. The conclusion should be smoothed because the mechanism has not been revealed.

7. Lines 201-222. It is not clear which is the relationship between SLC7A14 and TCDCA. Is TCDCA that regulates lipolysis and SLC7A14 plays the role of controlling TCDCA levels or SLC7A14 itself mediates lipolysis? Some clarifications must be done also in following parts (see below).

8. Lines 225-249. It seems that POMC SLC7A14 induces changes of expression of the transporter for TCDCA in the intestine. Authors speculate that POMC SLC7A14 acts on/via PPR-alpha (intestinal?). However, given that PPR-alpha is the regulator of ASBT expression, it results difficult to me understanding how this could occur at molecular level. This mechanism should be investigated further.

9. Lines 251- 267. Removal of ganglion indicates an action of the sympathetic nerves on expression of the ASBT transporter; however, this may be a very broad effect on many other systems, and it depends on ablation of nerves not strictly on SLC7A14. This is not the appropriate approach to demonstrate the hypothetical SLC7A14/ASBT axis. Therefore the next section cannot be titled “The SLC7A14-induced changes in intestinal sympathetic afferent nerves.....”.

10. 274-275. mTORC2 and mTORC1 are quite different in localization and composition. Thus, it is not straightforward that if SLC7A14 has effects on mTORC2 it will also interact with mTORC1. I would suggest that action/interaction with mTORC1 may be suggested by the lysosomal localization of SLC7A14.

Discussion.

General comment: repetition of results must be eliminated from the discussion.

Specific comments:

1) Author asserts correctly that it is not clear how CNS could regulate WAT lipolysis through some metabolites. However, the sentence "SLC7A14 is a lysosomal membrane protein that is highly expressed in the hypothalamus....." is not the proper link between metabolites and SLC7A14; it must be removed since.

2) The following sentences (lines 332-336) need to be rewritten since authors do not demonstrate any novel function of SLC7A14, but only its involvement in changes of another transporter (ASBT) through a mechanism probably acting on mTORC1 that is not described at the molecular level.

3) Lines 338-340 and 390-391. The administration of TCDCA for obesity treatment may not have the expected benefit because the transporter ASBT is down-regulated under this physio-pathological state and, hence, administered TCDCA not absorbed.

Reviewer #3 (Remarks to the Author):

This study investigated the possible role of hypothalamic SLC7A14 in the age-induced lipolysis reduction in mouse models. The concept of the study and some of the findings are indeed interesting. Below are some specific feedback points that require consideration to improve the understanding of the work.

1. It is suggested to share the raw LC-MS data via public repository such as Metabolomics Workbench or MetaboLights.

2. In the abstract, the authors shall spell out ASBT

3. Untargeted metabolomics:

There isn’t any information on the LC and mass spec setting for untargeted analysis and targeted analysis.

How much sample was injected for the LC-MS analysis?

What is the LC flow rate?

What is the LC gradient used, what are the beginning condition, and how the mobile phases evaluate with time?

What is the mass scan range, resolution?

What are the internal standards and chemicals used?

Are there any QC samples?

Is the LC-MS/MS system conditioned before analysis?

Have you evaluated the LC-MS/MS system suitability before running the study samples?

Were samples injected in the LC-MS/MS system in a randomized order?

Idem for the NMR analysis.

4. In the abstract, the authors need to clarify how many mice in each group. This information also needs to mention in the Methods.

5. It is suggested to refine the Discussion.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

What are the noteworthy results?
The study evaluated the mechanisms of regulation of lipolysis during ageing. Authors suggest that during ageing, the levels of POMC SLC7A14 are reduced leading to the activation of mTORC1. This generates a sympathetic signal that reduces the expression of ASBT in enterocytes, thus, reducing reabsorption of TCDCA. Authors suggest that TCDCA acts through TGR5 in WAT to induce lipolysis. Despite the undisputed importance of the finding, there are several gaps that should be filled in before the whole story is ready.

Will the work be of significance to the field and related fields? How does it compare to the established literature? If the work is not original, please provide relevant references.
It is original, and it is important to the field.

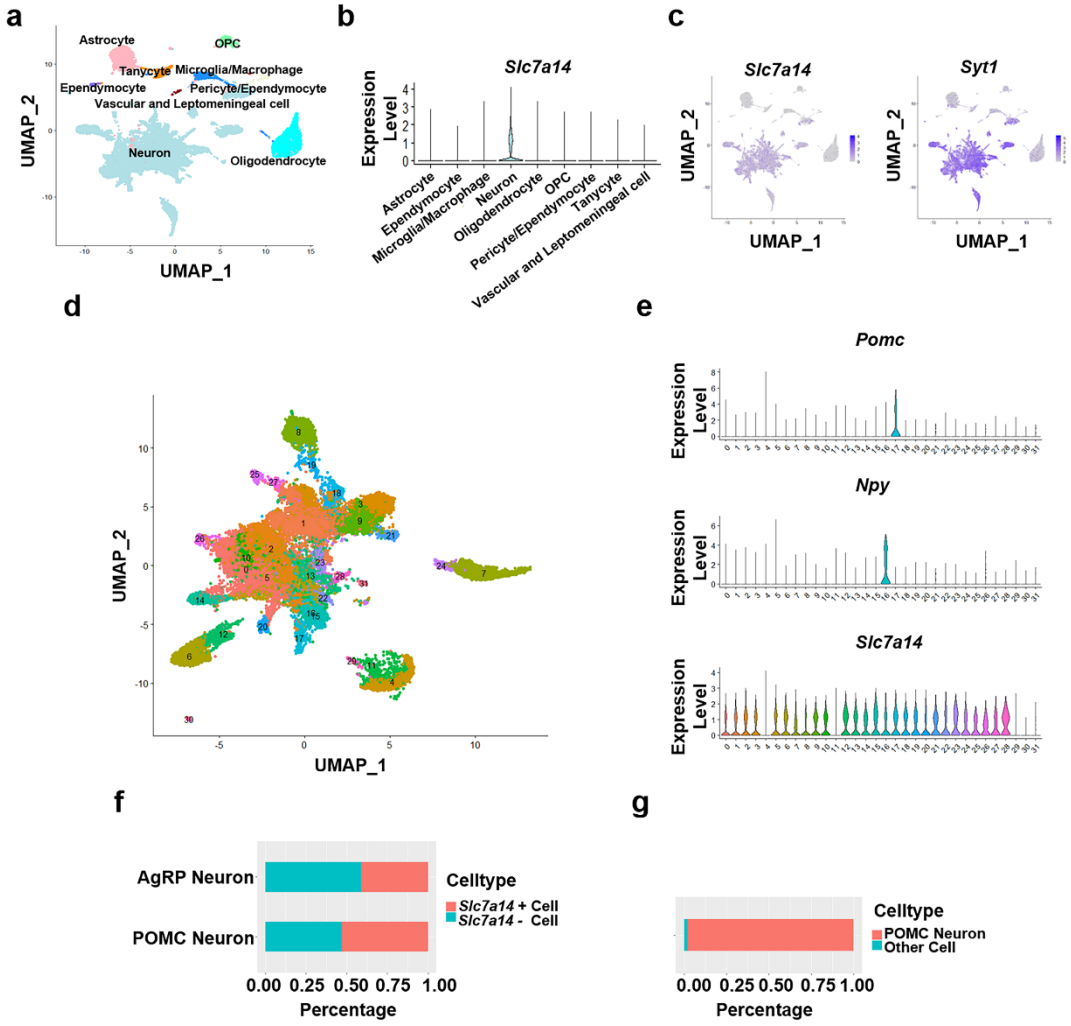
Does the work support the conclusions and claims, or is additional evidence needed?
There are a considerable number of missing pieces. There is a need for quite a lot of additional evidence.

Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision?
Yes, as follows:

The characterization of SLC7A14 expression and distribution in the brain, and particularly in the hypothalamus was very superficial. Authors should determine cell specific expression of SLC7A14 using single-cell RNA seq. This is particularly important because it is unclear whether SLC7A14 is expressed in AgRP and POMC neurons in WT mice. Out of the cells expressing SLC7A14 in the hypothalamus how many are POMC?

Our response:

We appreciate it very much for the reviewer's suggestion. In response to the reviewer's inquiry, we explored the cell specific expression of SLC7A14 in the hypothalamus using single-cell RNA seq (GSE188646). We found that SLC7A14 was mainly expressed in neurons defined by co-expression with *Syt1* (Response Figure 1a-1c). Further analysis showed that SLC7A14 was widely expressed in the neurons of hypothalamus, including AgRP and POMC neurons (Response Figure 1d-1f). Furthermore, 2.08% cells expressing SLC7A14 in hypothalamus are POMC neurons (Response Figure 1g). We apologize for not including this data in our current manuscript, as we are exploring the cell specific expression of SLC7A14 in the hypothalamus in another study, we wish the reviewer could understand our concern.



Response Figure 1. Single-cell analysis of the hypothalamus
(a) Uniform Manifold Approximation and Projection (UMAP) plot of all 40,064 nuclei used for analysis. Clustering analysis revealed 9 broad categories of cell type identity.
(b) Violin plot showing the expression of *Slc7a14* in the 9 hypothalamic nuclei clusters.
(c) UMAP plots of all nuclei labeled for expression of *Syt1*, *Slc7a14*
(d) UMAP of all neuronal nuclei. Distinct clusters are identified by color.
(e) Violin plot showing the expression of *Pomc*, *Npy*, *Slc7a14* in the neuronal nuclei clusters.
(f) The percentage of *Slc7a14* expressing cells in POMC and AgRP neurons.
(g) The percentage of POMC neurons in out of the cells expressing SLC7A14 in the hypothalamus.
Data for a-g using single-cell RNA seq (GSE188646).

There is a tremendous gap of information between the effect of POMC SLC7A14 on TCDCA and the capacity of TCDCA to control lipolysis. I could not find any prior study that explored such mechanism. Considering that the central issue of the present study is, how the hypothalamus of ageing mice control lipolysis, authors should go deep into the mechanism involved in TCDCA-dependent regulation of HSL.

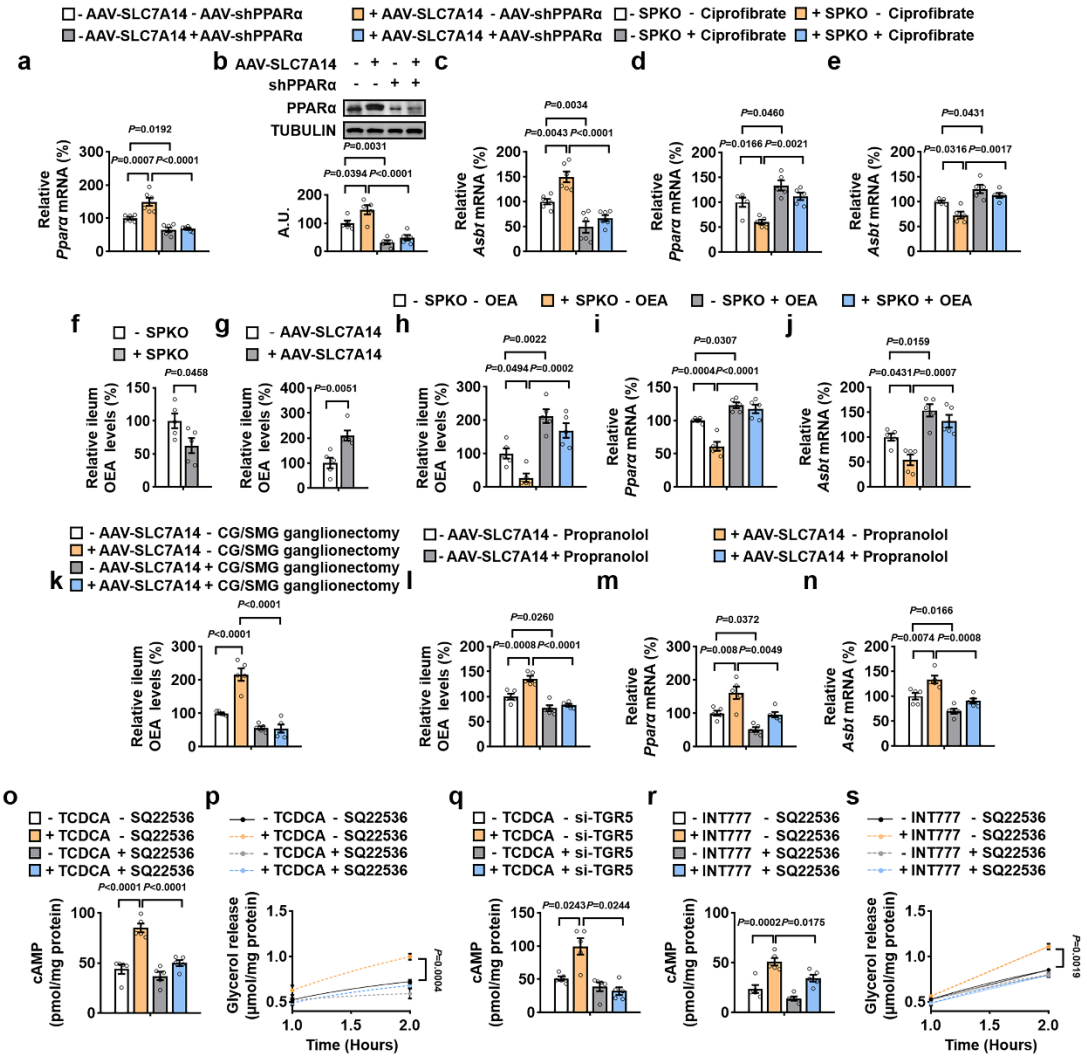
Our response:

We appreciate it very much for the reviewer's opinion. Regarding the effect of POMC SLC7A14 on TCDCA: in our previous study, we found that POMC SLC7A14-increased TCDCA content is dependent on intestinal ASBT, which is regulated by intestinal sympathetic afferent nerves. Although we found a correlation between PPARα and SLC7A14-increased ASBT expression, it is still unclear whether PPARα is involved in regulating SLC7A14-increased ASBT expression and how intestinal sympathetic afferent nerves regulate PPARα. To demonstrate the role of PPARα in this regulation, we injected SPOE mice with AAVs expressing small-hairpin RNA specific for PPARα (+ AAV-shPPARα) or EGFP fluorescent protein (-AAV- shPPARα) in ileum (Response Figure 2a and 2b). Knockdown of PPARα blocked POMC SLC7A14-increased ASBT expression (Response Figure 2c). Furthermore, we injected SPKO mice with ciprofibrate, a PPARα activator (Jung D et al. J Biol Chem. 2002 Aug 23;277(34):30559-66), and found that activation of PPARα rescued SLC7A14 knockout-decreased ASBT expression (Response Figure 2d and 2e). Next, we explored how PPARα expression was regulated by POMC SLC7A14. A previous study has reported that oleoylethanolamide (OEA), which is regulated by the intestinal sympathetic afferent nerves, is an endogenous high-affinity agonist of PPARα (Bowen KJ et al. Progress in lipid research vol. 67 (2017): 1-15). Consistently, we found that SLC7A14 could regulate OEA levels and injected SPKO mice with OEA rescued SLC7A14 knockout-decreased PPARα and ASBT expression (Response Figure 2f-2j). We further explored the involvement of sympathetic afferent nerves in regulating the intestinal OEA levels by POMC SLC7A14. We found that removal of the CG-SMG inhibited intestinal OEA levels and blocked POMC SLC7A14-promoted the increase of OEA levels (Response Figure 2k). Moreover, SPOE mice were injected with propranolol, a β-adrenergic receptor inhibitor (Xiao F et al. Nat Commun. 2023 May 2;14(1):2523), blocked POMC SLC7A14-promoted OEA levels, PPARα and ASBT expression (Response Figure 2l-2n). These results strongly suggest that OEA mediates POMC SLC7A14-activated intestinal sympathetic afferent nerves via regulating intestinal PPARα.

Regarding the capacity of TCDCA to control lipolysis: It is previously shown that TCDCA functions via TGR5 to increase the levels of cAMP (Qi YC et al. Chin J Nat Med. 2020 Dec;18(12):898-906), which is a key upstream of HSL (Grabner GF et al. Nat Metab. 2021 Nov;3(11):1445-1465). Thus, we speculated that TCDCA might regulate cAMP levels via TGR5, thereby regulating lipolysis. For this purpose, primary adipocytes were treated with TCDCA and SQ22536, a cAMP inhibitor (Kim SH et al. Blood. 2012 Dec 6;120(24):4892-902). As expected, SQ22536 treatment blocked TCDCA increased-cAMP levels and glycerol release in primary adipocytes (Response Figure 2o-2p). Using double-stranded siRNA targeting mouse TGR5, we showed that the effect of TCDCA on lipolysis depends on TGR5 (Response Figure 2q). Moreover, the stimulating effect of a TGR5 agonist INT777 (Thomas C et al. Cell Metab. 2009 Sep;10(3):167-77) on cAMP levels and glycerol release were blocked by SQ22536 (Response Figure 2r-2s). These results strongly suggest TGR5 mediates TCDCA-

stimulated lipolysis via regulating cAMP levels.

This information has been added to Results (page 10-14) in the revised manuscript.



Response Figure 2. TGR5 mediates TCDCA-stimulated lipolysis via regulating cAMP levels

(a, d, i and m) The *Pparα* mRNA levels in ileum by RT-PCR.

(b) PPARα and TUBULIN proteins in ileum by western blotting (top) and quantified by densitometric analysis (bottom); A.U.: arbitrary units.

(c, e, j and n) The *Asbt* mRNA levels in ileum by RT-PCR.

(f, g, h, k and l) The OEA levels of ileum.

(o, q and r) The cAMP levels of primary white adipocytes.

(p and s) Glycerol release assays in primary white adipocytes.

Studies for a-c, g, k-n were conducted using POMC Cre mice receiving AAVs expressing mCherry (- AAV-SLC7A14) or SLC7A14 (+ AAV-SLC7A14) in ARC, and the ileum of mice was injected with AAVs expressing shPPARα (+ AAV-shPPARα) or EGFP(- AAV-shPPARα) in a-c, and mice were treated with sham surgery (- CG/SMG ganglionectomy) or surgical removal of celiac-superior mesenteric ganglion (+ CG/SMG ganglionectomy) in k, and mice were injected with propranolol for 30 mins

before euthanized in l-n. Studies for d, e, f and h-j were conducted using 5-month-old control mice (- SPKO) or mice with SLC7A14 deletion in POMC neurons (+ SPKO), and mice were given ciprofibrate (10 mg/kg, MCE) once daily for 1 weeks in d-e, and mice were given OEA (10 mg/kg, aladdin), once daily for 2 weeks in h-j; o-p were conducted using primary white adipocytes treated with or without 100 μM TCDCA in the presence or absence of 100 μM SQ22536 for 4 h; q were conducted using primary white adipocytes transfected with double-stranded siRNA targeting mouse TGR5 for 72 h, followed by treating with control vehicle (- TCDCA) or 100 μM TCDCA (+ TCDCA) for 4 h; r-s were conducted using primary white adipocytes treated with or without 30 μM INT777 in the presence of control vehicle or 100 μM SQ22536 for 2 h. Data are expressed as the mean ± SEM, with individual data points. Data were analyzed by ordinary two-way ANOVA with Tukey's multiple comparisons test (a-e, h-n, o and q-r), or two-tailed unpaired Student's t test (f and g), or two-way RM ANOVA with Geisser-Greenhouse's correction (p, s).

Since authors propose that TCDCA acts through TGR5 to control lipolysis in WAT, why it is not acting through BAT TGR5 to stimulate BAT thermogenesis, a well know mechanism. What provides this tissue specificity?

Our response:

We are very grateful to the reviewer for pointing out this issue. Based on previous studies, the activation of TGR5 in BAT stimulates thermogenesis (Ding L et al. Acta Pharm Sin B. 2021 Jun;11(6):1541-1554). However, besides extensively studied TGR5, there are other BA-responsive receptors, such as pregnane X receptor (PXR), constitutive androstane receptor (CAR), vitamin D3 receptor (VDR), formyl-peptide receptors (FPRs), sphingosine 1-phosphate receptor 2 (S1PR2), and muscarinic acetylcholine receptors (mAChRs), respond to bile acid stimulation and exert different effects in various tissues and cell types (Perino A et al. Physiol Rev. 2021 Apr 1;101(2):683-731). For example, VDR has the opposite effect on BAT thermogenesis compared with TGR5. Overexpression of VDR in the brown adipose tissue decreases the expression of UCP1 (Xu Y et al. Eur J Pharmacol. 2019 Dec 15;865:172761). The expression of UCP1 is increased in the brown adipose tissues of VDR KO mice (Wong KE et al. Am J Physiol Endocrinol Metab. 2009 Apr;296(4):E820-8). Thus, in our study, TCDCA had no effect on BAT thermogenesis, possibly due to the role of other BA-responsive receptors, except TGR5, in brown adipose tissue. This possibility required to be investigated in the future.

This information has been added to Discussion (page 18) in the revised manuscript.

Authors suggest that the regulation of ASBT by POMC stimulus could be mediated by PPARa, but this hasn't been explored in depth.

Our response:

We appreciate it very much for the reviewer's pointing out this important issue.

Studies have shown that ASBT promoter, especially its untranscribed 5'-flanking region, can be activated by PPARα, and further study shows that DR1 element of ASBT can bind to the PPARα, and targeted mutagenesis of the DR1 motif abolished PPARα responsiveness (Jung D et al. J Biol Chem. 2002 Aug 23;277(34):30559-66), suggesting an important role of PPARα in the regulation of ASBT expression. In response, we explored the involvement of PPARα in POMC SLC7A14-increased ASBT expression. For this purpose, we injected SPOE mice with AAVs expressing small-hairpin RNA specific for PPARa (+ AAV-shPPARα) or EGFP fluorescent protein (- AAV-shPPARα) in ileum and found knockdown of PPARα blocked POMC SLC7A14-increased ASBT expression (Response Figure 2a-2c). Furthermore, we injected SPKO mice with ciprofibrate, a PPARα agonist (Jung D et al. J Biol Chem. 2002 Aug 23;277(34):30559-66), and found that activation of PPARα rescued SLC7A14 knockout-induced decreased ASBT expression (Response Figure 2d and 2e). These results strongly suggest that POMC SLC7A14 regulates intestinal ASBT via PPARα (please also see our response to question No. 2).

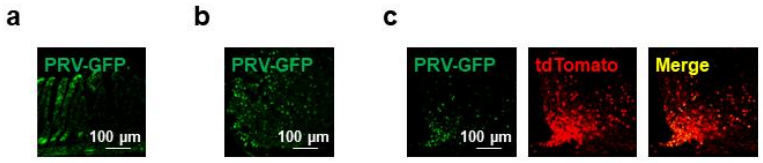
This information has been added to Results (page 12) in the revised manuscript.

Is the methodology sound? Does the work meet the expected standards in your field?
There are some experiments that are not convincing and additional evidence should be provided
Authors should use retrograde tracers to provide undisputed evidence for the effect of POMC SLC7A14 on gut sympathetic control of ASBT.

Our response:

We appreciate it very much for the reviewer's suggestion. In response to the reviewer's inquiry, we injected pseudorabies virus (BC-PRV-531-PRV-CAG-EGFP) into the ileum of POMC Cre mice (Response Figure 3a), which carrying Ai9 (tdTomato) reporter. After 6 days, a fluorescent signal of the PRV was seen in the CG-SMG and found to be merged with POMC neurons (Response Figure 3b-3c), indicating that POMC neurons can potentially deliver information to the ileum though intestinal sympathetic afferent nerves.

This information has been added to Results (page 13) in the revised manuscript.



Response Figure 3. Transsynaptic neuronal tracing of pathways descending from the POMC neuron to the ileum.
(a)Representative micrographs of the ileum wall demonstrating neurons expressing PRV (green).
(b)Representative micrographs of the CG-SMG demonstrating neurons expressing PRV

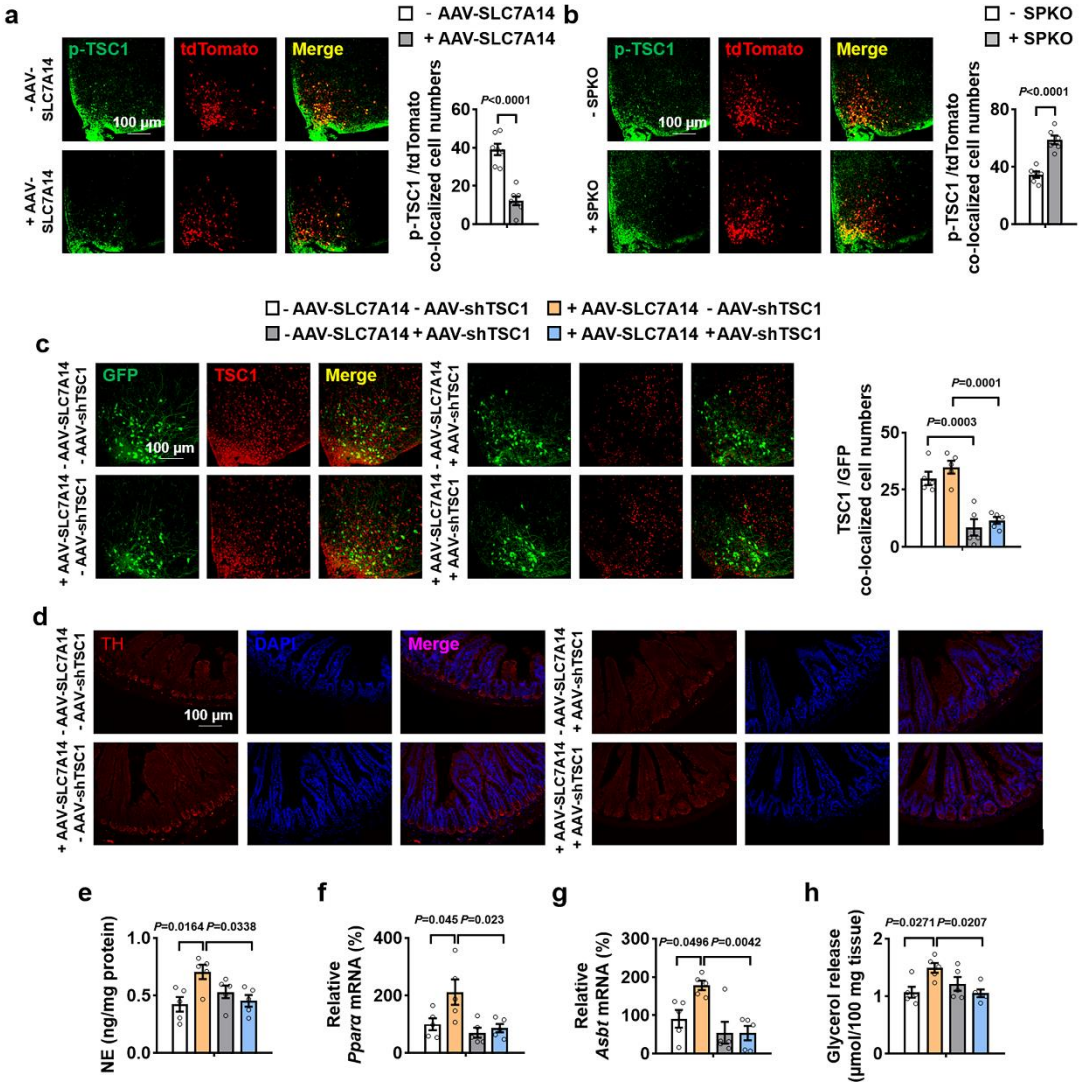
(green).
(c)Representative micrographs of the brain demonstrating neurons expressing PRV (green), tdTomato (red) and merge (yellow).
Studies for a-c were conducted using 5-month-old POMC Cre mice, which carrying Ai9 (tdTomato) reporter, injected with pseudorabies virus (BC-PRV-531-PRV-CAG-EGFP) for 6 days in ileum.

Exploring the POMC signaling mechanisms involved in the response to SLC7A14 using immunoblot of hypothalamic cells is not appropriate in the context of such a multi organic, systemic phenomenon.

Our response:

We are very grateful to the reviewer for pointing out this issue. In response, we examined the regulation of SLC7A14 on phosphorylation of TSC1 in POMC neurons. As predicted, overexpression of SLC7A14 in POMC neurons inhibited the phosphorylation of TSC1 and knockout of SLC7A14 enhanced its levels, which was consistent with the results observed in hypothalamic cells (Response Figure 4a-4b). Furthermore, knockdown of TSC1 in POMC neurons blocked POMC SLC7A14-promoted activation of intestinal sympathetic afferent nerves (Response Figure 4c-4e), intestinal PPARα and ASBT expression (Response Figure 4f-4g), and glycerol release in WAT (Response Figure 4h).

This information has been added to Results (page 15-16) in the revised manuscript.



Response Figure 4. POMC SLC7A14 regulates intestinal sympathetic afferent nerves though TSC1

(a and b) Immunofluorescence (IF) staining for tdTomato (red), p-TSC1 (green) and merge (yellow) in ARC sections (left), and quantification of p-TSC1 and tdTomato colocalized cell numbers (right).

(c) Immunofluorescence (IF) staining for GFP (green), TSC1 (red) and merge (yellow) in ARC sections (left), and quantification of TSC1 and GFP colocalized cell numbers (right).

(d) Immunofluorescence (IF) staining for TH (red).

(e) The NE levels of ileum.

(f) The mRNA levels of *Pparα* in ileum by RT-PCR.

(g) The mRNA levels of *Asbt* in ileum by RT-PCR.

(h) Glycerol release assays in sWAT.

Studies for a were conducted using 20-month-old POMC Cre mice receiving AAVs expressing mCherry (- AAV-SLC7A14) or SLC7A14 (+ AAV-SLC7A14) in ARC;b were conducted using 5-month-old control mice (- SPKO) or mice with SLC7A14 deletion in POMC neurons (+ SPKO); c-h were conducted using 20-month-old POMC

Cre mice receiving AAVs expressing mCherry (- AAV-SLC7A14) or SLC7A14 (+ AAV-SLC7A14) and AAVs expressing GFP (- AAV-shTSC1) or shTSC1(+ AAV-shTSC1) in ARC. Data are expressed as the mean ± SEM, with individual data points. Data were analyzed by two-tailed unpaired Student's t test (a and b), or ordinary two-way ANOVA with Tukey's multiple comparisons test (c, e-h).

Is there enough detail provided in the methods for the work to be reproduced?
Yes.

Additional suggestions
The introduction is far too long. It contains a number of basic definitions that should be removed. Authors should provide at most two or three paragraphs of background strictly related to the central question, and then a final paragraph for raising the hypothesis and objectives of the study.

Our response:
We agree with the reviewer's suggestion. In response, we have removed the basic definitions and simplified the introduction of manuscript.

This information has been updated in Introduction (page 3-5) of revised manuscript.

Reviewer #2 (Remarks to the Author):

Jiang et al. describe an interesting study on the involvement of SLC7A14 on the onset of age-related obesity. SLC7A14 belongs to a membrane transporter family, hence it is expected to play a transport function in cell (lysosomal) membrane. However, the author study is limited to a description of relationships among SLC7A14 expression/knocking out and regulation of obesity insurgence in mice through a pathway involving an intestinal TCDCA transporter. The work is well conducted on the experimental point of view but has two major flaws: i) no insights into the function of SLC7A14 are provided and correlated to the described role; ii) no molecular description of the mechanism by which a central nervous system district acts on intestinal absorption of a metabolite, as the same authors state at the end of discussion. At least one of the two issues should be dealt with and elucidated. Then, the manuscript should be changed in its structure, accordingly.

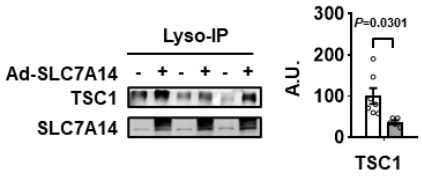
Our response:
We appreciate it very much for the reviewer's suggestion. Regarding the function of SLC7A14 are provided and correlated to the described role: In our previous study, we show that the subcellular localization of SLC7A14 is on lysosomal membrane and can regulate mTORC2 signaling pathway as a GABA transporter (Jiang X et al. Cell Rep. 2023 Jan 31;42(1):111984). mTOR is composed of two complexes including mTORC1 and mTORC2 with different functions and structures (Kim J et al. Nat Cell

Biol. 2019 Jan;21(1):63-71). In our present study, we found that SLC7A14 regulate mTORC1 signaling pathway via TSC1, which is a negative regulator of mTORC1 (Lee DF et al. Cell. 2007 Aug 10;130(3):440-55). Because SLC7A14 is located on lysosomal membrane (Jiang X et al. Cell Rep. 2023 Jan 31;42(1):111984) and the lysosomal translocation of TSC1 is shown to inactivate mTORC1 (Demetriades C et al. Cell. 2014 Feb 13;156(4):786-99; Menon S et al. Cell. 2014 Feb 13;156(4):771-85; Fitzian K et al. Mol Cell. 2021 Jul 1;81(13):2705-2721.e8), we speculated that SLC7A14 may have effect on lysosomal translocation of TSC1. To test this possibility, we isolated lysosomes from control or SLC7A14 overexpressing cells, and examined the lysosomal expression of TSC1. As expected, SLC7A14 promoted TSC1 translocation to lysosomal membrane (Response Figure 5). However, SLC7A14 may also function as a transporter to regulate mTORC1 signaling pathway, as we previously showed that SLC7A14 could increase lysosomal GABA levels (Jiang X et al. Cell Rep. 2023 Jan 31;42(1):111984), and furthermore, lysosomal amino acid played important role in regulating mTORC1 activity (Zoncu R et al. Science. 2011 Nov 4;334(6056):678-83; Wyant GA et al. Cell. 2017 Oct 19;171(3):642-654.e12). This possibility has not been investigated in the current study, therefore we modified the description related to its transport function in Discussion as the following: SLC7A14 inhibits mTORC1 signaling pathway may though regulating lysosomal translocation of TSC1, and/or via its transport function. However, more evidence will be need to support these possibilities.

Regarding the mechanism by which a central nervous system district acts on intestinal absorption of a metabolite: In response, to investigate whether POMC neurons could potentially deliver information to the ileum though intestinal sympathetic afferent nerves, we injected pseudorabies virus into the ileum of mice, and found a fluorescent signal of the PRV was seen in the ileum, CG-SMG and POMC neurons (Response Figure 3). Furthermore, we explored the involvement of intestinal sympathetic afferent nerves in POMC SLC7A14-increased ASBT expression via removal of the CG-SMG or injecting propranolol, a β-adrenergic receptor inhibitor (Xiao F et al. Nat Commun. 2023 May 2;14(1):2523) in SPOE mice. We found inhibition of intestinal sympathetic afferent nerves blocked POMC SLC7A14-increased ASBT expression (Figure 6o and Response Figure 2l-2n). Next, we investigated the mechanism by which POMC SLC7A14 regulates intestinal sympathetic afferent nerves regulation of ASBT. Oleoylethanolamide (OEA), which is regulated by sympathetic innervation of the gut (Fu J et al. J Neurosci. 2011 Apr 13;31(15):5730-6), is a small-intestinal factor. It is an endogenous high-affinity agonist of PPARα (Bowen KJ et al. Progress in lipid research vol. 67 (2017): 1-15). We showed that POMC SLC7A14 could regulate OEA levels (Response Figure 2f-2g and 2k-2n) and injected SPKO mice with OEA blocked SLC7A14 knockout-decreased PPARα and ASBT expression (Response Figure 2h-2j). Furthermore, we injected SPOE mice with AAVs expressing small-hairpin RNA specific for PPARa (+ AAV-shPPARα) or EGFP fluorescent protein (-AAV- shPPARα) in ileum. Knockdown of PPARα in SPOE mice ileum blocked POMC SLC7A14-increased intestinal ASBT expression (Response Figure 2a-2c). SPKO mice were injected with ciprofibrate, a PPARα activator (Jung D et al. J Biol

Chem. 2002 Aug 23;277(34):30559-66), and found that activation of PPARα rescued SLC7A14 knockout-decreased ASBT expression (Response Figure 2d and 2e). These results strongly suggest POMC SLC7A14 regulates intestinal sympathetic afferent nerves, then regulating OEA levels, thereby regulating intestinal PPARα expression, thus regulating ASBT expression and TCDCA levels (please also see our response to question No. 2 from the first reviewer).

This information has been added to Results (page 12-14) and Discussion (page 19) in the revised manuscript.



Response Figure 5 The TSC1 translocation on lysosomal membrane
Studies were using stable cell lines expressing the transmembrane protein 192 (TMEM192) fused to 3XFLAG epitopes, and lysosomes were then immunopurified with anti-FLAG magnetic beads.

Specific comments:
Introduction must be rewritten trying to describe what is known around the subject (SLC7A14) and how it is connected with the other players (mTOR ect.) arriving to the final hypothesis. Major flows of introduction are listed below.
1. Only mTORC1 is described but not mTORC2 that, after, appear to be involved in SLC7A14 action.

Our response:

We agree with the reviewer's opinion. mTOR complex contains two components including mTORC1 and mTORC2. In addition to mTORC1, mTORC2 also plays an important role in age-related obesity. As suggested, we have added information related to mTORC2 in Introduction as the following "mTOR is a master regulator of cell growth and metabolism that forms two complexes with different structures and functions, namely, mTORC1 and mTORC2. Both mTORC1 and mTORC2 signaling pathway are shown to be related to age-related obesity (Yang SB et al. Neuron. 2012 Aug 9;75(3):425-36; Kocalis HE et al. Mol Metab. 2014 Feb 19;3(4):394-407). For example, recent studies have shown that the activation of mTOR in hypothalamic POMC neurons of aged mice results in the silencing of POMC neurons due to the activity of the ATP-sensitive potassium (KATP) channel (Yang SB et al. Neuron. 2012 Aug 9;75(3):425-36). Injection of the mTOR inhibitor rapamycin into the brain of aging mice causes weight loss. Loss of mTORC2 in POMC neuron increased fat mass of aged mice (Kocalis HE et al. Mol Metab. 2014 Feb 19;3(4):394-407)".

This information has been added to Introduction (page 4) in the revised manuscript.

2. Lines 66-70 can be removed since are useless for readers in the frame of the manuscript.

Our response:

We agree with the reviewer's suggestion. As suggested, we have removed the description of lines 66-70.

3. Line 75: TSC1 appears after description of TSC (explain what TSC1 is for non-expert readers).

Our response:

We agree with the reviewer's suggestion. As suggested, we have added the description of TSC1.

This information has been added to Introduction (line 81-82) in the revised manuscript.

4. Lines 78-79 seems in contrast with 75-76. Rephrase

Our response:

We agree with the reviewer's suggestion. In response to the reviewer's inquiry, we rephrased the sentence as the following "Both mTORC1 and mTORC2 signaling pathway are shown to be related to age-dependent obesity".

This information has been added to Introduction (line 74-75) in the revised manuscript.

5. Lines 85-88: the sentences seem to not be connected to the following one "solute carrier....". Which is the connection between SLC7A14 and the metabolites that can mediate the effects of the CNS on lipolysis? GABA/arginine may be involved?

Our response:

We appreciate it very much for the reviewer's pointing out this critical issue. The connection between central SLC7A14 and intestinal TCDCA absorption is mediated by sympathetic signal. The sentences (lines 85-88) are intended to give some background information about lipolysis control, particularly metabolites as new regulator gained much more attention recently. However, adding this information between hypothalamic mTOR and SLC7A14 seems to be misleading and inappropriate, we have moved this information to the first paragraph, following the introduction of hypothalamic control of lipolysis.

This information has been added to Introduction (page 2-4) in the revised manuscript.

6. Lines 95-98 are again not well connected. Which is the message?

Our response:

Thanks for the reviewer for pointing out this important issue. The sentences in lines 95-98 are intended to introduce some known functions of SLC7A14. We have rephrased the lines 95-98 with "It has been indicated in the regulation of retina function, auditory neuropathy, as well as insulin resistance, demonstrated by using global or tissue specific knockout mice. SLC7A14 is expressed in different tissues, most abundantly in the CNS, but its central role is unclear."

This information has been added to Introduction (page 4-5) in the revised manuscript.

7. From line 99 mTORC2 appears which had not been mentioned before but now may be the target of the subject of the manuscript.

Our response:

We agree with the reviewer's suggestion. We agree with the reviewer's opinion. mTORC2 plays an important role in regulation of age-related obesity. As suggested, we have added more information related to mTORC2 in Introduction before line 99, and revised line 99 with "Based on the fact that mTORC2 activity is regulated by SLC7A14 and its subcellular location is on lysosome, where mTORC1 activity significantly affected, it is possible that SLC7A14 functions as upstream regulator for mTOR".

This information has been added to Introduction (page 5) in the revised manuscript.

8. Lines 101-104 put forward a well-defined hypothesis that is difficult to derive from the previous sentences and uncertainties.

Our response:

We agree with the reviewer's suggestion. The transport function of SLC7A14 has not been involved in current research, but we have used a lot of sentences to describe it. In response, to put forward a well-defined hypothesis, we have removed the description of the transport function of SLC7A14 in the introduction and provided the sentences of background strictly related to the central question, as the following, "Based on the fact that mTORC2 activity is regulated by SLC7A14 (Jiang X et al. Cell Rep. 2023 Jan 31;42(1):111984) and its subcellular location is on lysosome (Jiang X et al. Cell Rep. 2023 Jan 31;42(1):111984), where mTORC1 activity significantly affected (Zoncu R et al. Science. 2011 Nov 4;334(6056):678-83; Wyant GA et al. Cell. 2017 Oct 19;171(3):642-654.e12), it is possible that SLC7A14 functions as upstream regulator for mTOR. Because the hypothalamic mTOR signaling pathway is strongly associated with age-dependent obesity, we speculated that hypothalamic SLC7A14 may play an important role in the aging-related increase in the fat mass via mTOR pathway. The aim of the current study was to investigate this hypothesis and explore the underlying

mechanisms".

This information has been added to Introduction (page 4-5) in the revised manuscript.

Results.
1. Line 113 "highly expressed" is not appropriate since there is not a control of "normal" expression. It should be stated as example: "expression in HPO and HY is higher than expression in ...".

Our response:
We agree with the reviewer's suggestion. As suggested, we have rewritten the sentence as the following, " the expression of SLC7A14 in hippocampus (HPO) and hypothalamus (HY) is higher than expression in cortex (COR), amygdala (AMY) and cerebellum (CB)".

This information has been added to Results (page 5) in the revised manuscript.

2. Legend of fig S2b is not clear: what - 22M means? Both 8W and 22M old mice? May be there is an error in the indication.

Our response:
Thanks for the reviewer for pointing out this important issue. We are sorry for the confusion. "- 22M" means 2 months old mice and "+ 22M" means 22 months old mice. To make it more clearly, we have changed the indication of "- 22M" with "2 M" in Fig S1b and revised the Figure Legends of Fig S1b.

This information has been added to Extended Figures and Extended Figure legends (page 57-59) in the revised manuscript.

3. The same problem is present in Fig. 1 legend.

Our response:
Thanks for the reviewer for pointing out this important issue. We are sorry for the confusion. In response, we have changed the indication of "- 22M" with "2 M" in Fig. 1 and revised the Figure Legends of Fig. 1.

This information has been added to Figures and Figure legends (page 38-39) in the revised manuscript.

4. Line 131 please report the extended name of enzyme (Hormone sensitive lipase?)

Our response:
Thanks for the reviewer for pointing out this important issue. As suggested, we

have added the extended name of HSL with hormone sensitive lipase.

This information has been added to Results (page 6) in the revised manuscript.

5. Line 202, the sentence lacks something or the comma must be eliminated.

Our response:

We appreciate it very much for the reviewer's suggestion. In response, we modified the sentence as the following, "To investigate the mechanisms underlying SLC7A14-increased WAT lipolysis, we performed serum metabolomics in control and SPOE mice serum."

This information has been added to Results (page 10) in the revised manuscript.

6. Lines 221-222. The conclusion should be smoothed because the mechanism has not been revealed.

Our response:

We agree with the reviewer's suggestion. In response, we modified the sentence as the following, "These results strongly suggest that the decreased TCDCA may account for SPKO-impaired WAT lipolysis, and the effect of TCDCA is mediated by upregulating cAMP levels through TGR5."

This information has been added to Results (page 11) in the revised manuscript.

7. Lines 201-222. It is not clear which is the relationship between SLC7A14 and TCDCA. Is TCDCA that regulates lipolysis and SLC7A14 plays the role of controlling TCDCA levels or SLC7A14 itself mediates lipolysis? Some clarifications must be done also in following parts (see below).

Our response:

Thanks for the reviewer for pointing out this important issue. We are sorry for the confusion. Our study demonstrates an important role for SLC7A14/mTORC1 signaling in POMC neurons in regulating age-induced reduction in lipolysis. This process depends on sympathetic signal that reduces intestinal ASBT expression in enterocytes and the subsequent intestinal reabsorption of TCDCA. TCDCA then stimulate WAT lipolysis via TGR5. In response, we have explained the relationship between SLC7A14 and TCDCA in the revised manuscript, and modified the title for this paragraph.

This information has been added to Results (page 9-11) in the revised manuscript.

8. Lines 225-249. It seems that POMC SLC7A14 induces changes of expression of the transporter for TCDCA in the intestine. Authors speculate that POMC SLC7A14 acts on/via PPR-alpha (intestinal?). However, given that PPR-alpha is the regulator of ASBT

expression, it results difficult to me understanding how this could occur at molecular level. This mechanism should be investigated further.

Our response:

Thanks for the reviewer for pointing out this important issue. We are sorry for the confusion. In our study, we speculated that POMC SLC7A14 regulates ASBT via intestinal PPARα, as it has been shown that there is a binding site for PPARα in the promoter of ASBT (Jung D et al. J Biol Chem. 2002 Aug 23;277(34):30559-66). To further verify involvement of PPARα in POMC SLC7A14-increased ASBT expression, we injected SPOE mice with AAVs expressing small-hairpin RNA specific for PPARα (+ AAV-shPPARα) or EGFP fluorescent protein (-AAV-shPPARα) in ileum and found knockdown of PPARα rescued POMC SLC7A14-increased ASBT expression (Response Figure 2a-2c). Furthermore, we injected SPKO mice with ciprofibrate, a PPARα agonist (Jung D et al. J Biol Chem. 2002 Aug 23;277(34):30559-66), and found that activation of PPARα rescued SLC7A14 knockout-decreased ASBT expression (Response Figure 2d and 2e). These results provided important evidence for PPARα in the regulation of ASBT. (Please also see our response to question No. 2 from the first reviewer).

This information has been added to Results (page 12) in the revised manuscript.

9. Lines 251- 267. Removal of ganglion indicates an action of the sympathetic nerves on expression of the ASBT transporter; however, this may be a very broad effect on many other systems, and it depends on ablation of nerves not strictly on SLC7A14. This is not the appropriate approach to demonstrate the hypothetical SLC7A14/ASBT axis. Therefore the next section cannot be titled "The SLC7A14-induced changes in intestinal sympathetic afferent nerves…..".

Our response:

We appreciate it very much for the reviewer's suggestion. In response, we modified the title as the following, "POMC SLC7A14 regulates ASBT expression possibly via intestinal sympathetic afferent nerves". In our revised manuscript, to investigate the involvement of intestinal sympathetic afferent nerves in hypothetical SLC7A14/ASBT axis, we injected pseudorabies virus into the ileum of mice, and found a fluorescent signal of the PRV was seen in the ileum, CG-SMG and POMC neurons (Response Figure 3), indicating that POMC neurons can potentially deliver information to the ileum though intestinal sympathetic afferent nerves. Furthermore, we injected SPOE mice with propranolol, a β-adrenergic receptor inhibitor (Xiao F et al. Nat Commun. 2023 May 2;14(1):2523), and found blocking β-adrenergic receptor rescued POMC SLC7A14-promoted intestinal PPARα and ASBT expression (Response Figure 2c-2e). These results further suggest intestinal sympathetic afferent nerves plays an important role in regulating hypothetical SLC7A14/ASBT axis. (please also see our response to question No. 2 and No.3 from the first reviewer).

This information has been added to Results (page 13-14) in the revised manuscript.

10. 274-275. mTORC2 and mTORC1 are quite different in localization and composition. Thus, it is not straightforward that if SLC7A14 has effects on mTORC2 it will also interact with mTORC1. I would suggest that action/interaction with mTORC1 may be suggested by the lysosomal localization of SLC7A14.

Our response:

We agree with the reviewer's opinion. It will be more reasonable to predict the involvement of mTORC1 by the localization of SLC7A14. Thus, we have modified the sentence as the following, "Because mTORC1 activity is regulated at lysosome, where SLC7A14 is localized, we speculated that mTORC1 might be involved in the regulatory effect of SLC7A14".

This information has been added to Results (page 14) in the revised manuscript.

Discussion.
General comment: repetition of results must be eliminated from the discussion.

Our response:

We agree with the reviewer's suggestion. As suggested, we have removed the repetition of results from the Discussion.

Specific comments:
1) Author asserts correctly that it is not clear how CNS could regulate WAT lipolysis through some metabolites. However, the sentence "SLC7A14 is a lysosomal membrane protein that is highly expressed in the hypothalamus....." is not the proper link between metabolites and SLC7A14; it must be removed since.

Our response:

We agree with the reviewer's suggestion. As suggested, we have removed the sentence.

2) The following sentences (lines 332-336) need to be rewritten since authors do not demonstrate any novel function of SLC7A14, but only its involvement in changes of another transporter (ASBT) through a mechanism probably acting on mTORC1 that is not described at the molecular level.

Our response:

We agree with the reviewer's suggestion. As suggested, we have rewritten lines 332-336 as the following, "In this study, we found that the expression of POMC SLC7A14 are reduced that leading to the activation of mTORC1 during ageing. This generates a sympathetic signal that reduces the expression of ASBT in enterocytes and the subsequent reabsorption of TCDCA. TCDCA then induces WAT lipolysis via TGR5.

Furthermore, we identified the underlying mechanism of the regulation of the mTORC1 signaling pathway by SLC7A14."

This information has been added to Discussion (page 17) in the revised manuscript.

3) Lines 338-340 and 390-391. The administration of TCDCA for obesity treatment may not have the expected benefit because the transporter ASBT is down-regulated under this physio-pathological state and, hence, administered TCDCA not absorbed.

Our response:

Thanks for the reviewer for pointing out this important issue. As suggested, we have deleted Lines 338-340 and 390-391.

Reviewer #3 (Remarks to the Author):

This study investigated the possible role of hypothalamic SLC7A14 in the age-induced lipolysis reduction in mouse models. The concept of the study and some of the findings are indeed interesting. Below are some specific feedback points that require consideration to improve the understanding of the work.

1. It is suggested to share the raw LC-MS data via public repository such as Metabolomics Workbench or MetaboLights.

Our response:

We agree with the reviewer's suggestion. In response, we have uploaded the raw LC-MS data on MetaboLights (MTBLS9872).

This information has been added to Methods (page 29-30) in the revised manuscript.

2. In the abstract, the authors shall spell out ASBT

Our response:

Thanks for the reviewer for pointing out this important issue. As suggested, we have added the extended name of ASBT, which is "apical sodium dependent bile acid transporter".

This information has been added to Abstract (page 2) in the revised manuscript.

3. Untargeted metabolomics:
There isn't any information on the LC and mass spec setting for untargeted analysis and targeted analysis.

Our response:

We appreciate it very much for the reviewer's suggestion. In response, we have added the information on the LC and mass spec setting for untargeted analysis and targeted analysis in method, as described in the following:

For untargeted metabolomics analysis, the eluents for the positive polarity mode were eluent A (0.1% FA in Water) and eluent B (Methanol) and the eluents for the negative polarity mode were eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (Methanol). The solvent gradient was set as the following: 2% B, 1.5 min; 2-100% B, 12.0 min; 100% B, 14.0 min;100-2% B, 14.1 min; 2% B, 17 min. The loading volume is 1 μL. The LC flow rate is 200 μL/min.

For targeted metabolomics analysis, using 0.01% formic acid in water (solvent A) and acetonitrile with 0.01% formic acid (solvent B) as the mobile phase for binary gradient elution. The binary elution gradient was 25%B to 40%B in 12 minutes and then 40% to 75%B in 14 min. The column was washed with 100% B for 3 min and equilibrated with 25% B for 7 min between injections. The loading volume is 5 μL. The LC flow rate is 200 μL/min.

The blank sample was used before analysis to elute the residue of the previous sample and balance the entire system. All samples were injected in the LC-MS/MS system in a randomized order.

This information has been added to Methods (page 29-30) in the revised manuscript.

How much sample was injected for the LC-MS analysis?

Our response:

For untargeted analysis, the loading volume is 1 μL. For targeted analysis, the loading volume is 5 μL. This information has been added to Methods (page 29-30) in the revised manuscript.

What is the LC flow rate?

Our response:

The LC flow rate is 200 μL/min. This information has been added to Methods (page 29) in the revised manuscript.

What is the LC gradient used, what are the beginning condition, and how the mobile phases evaluate with time?

Our response:

For untargeted analysis, the eluents for the positive polarity mode were eluent A (0.1% FA in Water) and eluent B (Methanol) and the eluents for the negative polarity mode were eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (Methanol). The solvent gradient was set as follows: 2% B, 1.5 min; 2-100% B, 12.0 min; 100% B, 14.0 min;100-2% B, 14.1 min; 2% B, 17 min.

For targeted analysis, using 0.01% formic acid in water (solvent A) and acetonitrile with 0.01% formic acid (solvent B) as the mobile phase for binary gradient elution. The binary elution gradient was 25%B to 40%B in 12 minutes and then 40% to 75%B in 14 min. The column was washed with 100% B for 3 min and equilibrated with 25% B for 7 min between injections.

This information has been added to Methods (page 29-30) in the revised manuscript.

What is the mass scan range, resolution?

Our response:

The scanning range is m/z 100-1500, the primary scanning resolution is 60000 fwhm, and the secondary scanning resolution is 15000 fwhm.

What are the internal standards and chemicals used?

Our response:

We didn't use any internal standards. We used the external standard method in targeted analysis.

Are there any QC samples?

Our response:

For untargeted analysis, the QC samples are made by mixing equal volumes of experimental samples. For targeted analysis, we didn't use QC samples.

Is the LC-MS/MS system conditioned before analysis?

Our response:

The blank sample was used before analysis to elute the residue of the previous sample and balance the entire system.

Have you evaluated the LC-MS/MS system suitability before running the study samples?

Our response:

For untargeted analysis, mouse serum is a routine sample that can be detected by mass spectrometry. For targeted analysis, we used standards to examine linear and dynamic range.

Were samples injected in the LC-MS/MS system in a randomized order?
Idem for the NMR analysis.

Our response:

Yes, samples were injected in the LC-MS/MS system in a randomized order. This information has been added to Methods (page 29-30) in the revised manuscript.

4. In the abstract, the authors need to clarify how many mice in each group. This information also needs to mention in the Methods.

Our response:

Thanks for the reviewer for pointing out this important issue. As suggested, we have clarified how many mice in each group in Figure legends and Methods.

5. It is suggested to refine the Discussion.

Our response:

We agree with the reviewer's opinion. In response, we have modified the description in Discussion as suggested.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

My concern #1 was the lack of cell-specific determination of SLC7A14 in the hypothalamus. As requested in the first round of review, authors performed the analysis and identified the sub-populations of cells expressing the target transcript. The data was shown in the letter to Reviewer; however, authors argue they don't want to publish this data in the present article because they are exploring the issue in detail. As a reviewer, my opinion is that this data is important for the quality of the present work, and I maintain my claim that it should be published in this article.

Reviewer #2 (Remarks to the Author):

Authors have dealt with my comments. Therefore the manuscript may be acceptable, in my opinion.

Reviewer #3 (Remarks to the Author):

1. The mentioned raw LC-MS data on MetaboLights is not accessible. The metabolomics data matrix is not included in the supplementary data either.

2. More details are needed for the metabolomics analysis to better support the findings and to improve the reproducibility of the study.

a. For the Cluster analysis in Fig.3a. Which data was used, untargeted metabolomics or targeted metabolomics or both? Which tool was used for the Cluster analysis and which

Cluster analysis method was used? How was the data normalized for the Cluster analysis? How was the annotation of metabolites performed?

b. How was the serum sample prepared for the untargeted metabolomics and targeted metabolomics?

c. The authors need to clarify how many mice samples were used for the untargeted metabolomics and targeted metabolomics.

d. What are the LC columns used for the untargeted metabolomics and targeted metabolomics, with which LC flow rate?

e. What are the mass spectrometers used for the untargeted metabolomics and targeted metabolomics, what are the mass scan rang, resolution and mass spec settings such as ion source temperature, nitrogen flow rate ...

f. Line 723: “Further validation via LC/MS/MS...”, what was the validation carried out?

g. A principal component analysis (PCA) plot with all samples including QC samples is recommended in the supplement data.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

My concern #1 was the lack of cell-specific determination of SLC7A14 in the hypothalamus. As requested in the first round of review, authors performed the analysis and identified the sub-populations of cells expressing the target transcript. The data was shown in the letter to Reviewer; however, authors argue they don't want to publish this data in the present article because they are exploring the issue in detail. As a reviewer, my opinion is that this data is important for the quality of the present work, and I maintain my claim that it should be published in this article.

Our response:

We appreciate it very much for the reviewer's suggestion. In response to the reviewer's inquiry, we have added the analysis data of cell-specific determination of SLC7A14 in the hypothalamus in the revised manuscript.

This information has been added to Results (page 7) in the revised manuscript.

Reviewer #2 (Remarks to the Author):

Authors have dealt with my comments. Therefore, the manuscript may be acceptable, in my opinion.

Our response:

We thank the reviewer very much for appreciating our efforts and the way in which we dealt with these issues.

Reviewer #3 (Remarks to the Author):

1. The mentioned raw LC-MS data on MetaboLights is not accessible. The metabolomics data matrix is not included in the supplementary data either.

Our response:

We are sorry for not including the raw LC-MS data in the previously revised manuscript, as our submitted raw LC-MS data (MTBLS9872) is still in the "In Curation" status, and the data on MetaboLights will not be obtained until June 8. However, as suggested, we have added the metabolomics data matrix to the Supplementary Data 1 in our revised manuscript. To help the reviewer for better visualizing the data, we have also included an excel file in response letter, as shown in Response Table 1.

This information has been added to Method (page 31 and 34) and Supplementary Data 1 in the revised manuscript.

Description:Data were obtained using untargeted metabolomics in controlm						
ompound_	Name	Formula	lecularWe	RT [min]	m/z	Control1
Com_1410	2-Hydroxy	C6 H12 O3	132.0788	4.182	131.0715	5813789
Com_595_	DL-4-Hydi	C9 H10 O4	182.0581	2.307	181.0508	11314437
Com_1268	3-Methylc	C7 H11 N (	157.0741	5.285	156.0669	8304068
Com_1746	D-Glucose	C6 H13 O9	260.0297	1.202	259.0224	5443365
Com_447_	Oleoyl-L-	C21 H41 O	436.2588	14.985	435.2514	49287212

Response Table 1 The raw data of untargeted metabolomics. Data were obtained using untargeted metabolomics in control mice (n = 5, labeled as control 1-5), SLC7A14 overexpressed mice (n = 4, labeled as SPOE 1-4) and quality control sample (n = 3, labeled as QC 1-3).

2. More details are needed for the metabolomics analysis to better support the findings and to improve the reproducibility of the study.

Our response:

We appreciate it very much for the reviewer's suggestion, as detailed information is important for determining the accuracy and reproducibility of the experimental data. In response, we have provided more detailed information required for each question.

However, there is one thing we would like to explain first. We confirmed the role of bile acids in mediating central SLC7A14 in lipolysis regulation by untargeted metabolomics analysis and LC-MS/MS measurement using individual bile acid as standards. Because the measurement was specific for bile acids, we considered that the LC-MS/MS assay is a "targeted bile acids measurement" and therefore described it as a "targeted metabolomics analysis" (in the Method section of the previously revised manuscript). The detailed information included was also for LC-MS/MS analysis. However, given the fact for the limited quantities and types of bile acids examined by LC-MS/MS, it not inappropriate to define the method as "targeted metabolomics analysis".

In response, we have deleted the word "targeted metabolomics analysis", and removed the description regarding experimental details to the part for "Bile acids measurement" in Method section. In addition, we have provided more detailed information required for untargeted metabolomics analysis, as well as LC-MS/MS analysis, as suggested by the reviewer. The response to each individual question is shown as below.

a. For the Cluster analysis in Fig.3a. Which data was used, untargeted metabolomics or targeted metabolomics or both? Which tool was used for the Cluster analysis and which Cluster analysis method was used? How was the data normalized for the Cluster analysis? How was the annotation of metabolites performed?

Our response:

In Fig. 3a, we performed enrichment analysis, but not cluster analysis, using untargeted metabolomics data (MTBLS9872). We are sorry for the writing error, which has been corrected in the revised manuscript (page 10). The data normalization was based on QC (Quality Control) samples. The metabolites were annotated using the KEGG database, HMDB database and LIPID MAPS database, which is a commonly used method for annotating metabolites (Gut. 2022 Jun;71(6):1203-1213). We applied univariate analysis (t-test) to calculate the statistical significance (P-value). The metabolites with VIP > 1 and P-value < 0.05 and fold change $\geqslant$ 2 or FC $\leqslant$ 0.5 were considered to be differential metabolites. We used a list of differential metabolites names to perform metabolite set enrichment analysis (MSEA) on MetaboAnalyst 5.0.

This information has been added to Results (page 10) and Method (page 31 and 34) in the revised manuscript.

b. How was the serum sample prepared for the untargeted metabolomics and targeted metabolomics?

Our response:

For the untargeted metabolomics: the serum samples (100 μL) were resuspended with prechilled 80% methanol and 0.1% formic acid by well vortex. Then the samples were incubated on ice for 5 min and centrifuged at 15,000 g, 4 °C for 20 min. Some of the supernatant was diluted to final concentration containing 53% methanol by LC-MS grade water. The samples were subsequently transferred to a fresh Eppendorf tube and then centrifuged at 15000 g, 4 °C for 20 min. Finally, the supernatant was used for metabolomics analysis.

As explained at the beginning, we did not perform targeted metabolomics. For LC-MS/MS quantification of differential bile acids: the serum samples (60 μL) were resuspended with 1 mL prechilled acetonitrile by well vortex, and incubated at 4 °C for 2 h, then centrifuged at 11,000 g, 4 °C for 20 min. Freeze drying and vacuum evaporation of the supernatant. Then the samples were redissolved in 50% methanol and injected into the LC-MS/MS system analysis.

This information has been added to Method (page 29-32) in the revised manuscript.

c. The authors need to clarify how many mice samples were used for the untargeted metabolomics and targeted metabolomics.

Our response:

For the untargeted metabolomics: there are 5 mice samples in control group and 4 mice samples in SLC7A14 overexpressed group. We have clarified the sample number in each group in figure legends of Fig. 3a.

As explained at the beginning, we did not perform targeted metabolomics. For LC-MS/MS quantification of differential bile acids: there are 5 mice samples in control group and 4 mice samples in SLC7A14 overexpressed group in Fig. 3b and Fig. S8d.

There are 5 mice samples in control group and 5 mice samples in SLC7A14 knockout group in Fig. 3c and Fig. S8e. We have clarified the sample number in each group in figure legends of Fig. 3b-3c and Fig. S8d-8e.

This information has been added to Figures and Figure legends (page 44-45) and Supplementary Information (page 71) in the revised manuscript.

d. What are the LC columns used for the untargeted metabolomics and targeted metabolomics, with which LC flow rate?

Our response:

For the untargeted metabolomics: samples were injected onto a Hypesil Gold column (100×2.1 mm, 1.9 μm) using a 17-min linear gradient at a flow rate of 0.2 mL/min.

As explained at the beginning, we did not perform targeted metabolomics. For LC-MS/MS quantification of differential bile acids: samples were injected onto a Thermo scientific Acclaim C30 column (2.1x250mm, 3 μm). The column flow rate was 0.2 mL/min.

This information has been added to Method (page 29-32) in the revised manuscript.

e. What are the mass spectrometers used for the untargeted metabolomics and targeted metabolomics, what are the mass scan rang, resolution and mass spec settings such as ion source temperature, nitrogen flow rate …

Our response:

For untargeted metabolomics: UHPLC-MS/MS analyses were performed using a Vanquish UHPLC system (ThermoFisher, Germany) coupled with an Orbitrap Q ExactiveTMHF-X mass spectrometer (Thermo Fisher，Germany). Data was acquired within the mass/charge ratio (m/z) range of 100 to 1500 Da. MS1 resolution was 60000 fwhm and MS2 resolution was 15000 fwhm. Exactive TMHF-X mass spectrometer was operated in negative polarity mode with spray voltage of 3.2 kV, capillary temperature of 320°C, sheath gas (N2) flow rate of 40 arb and aux gas (N2) flow rate of 10 arb. The metabolite assessment of samples was performed by untargeted metabolomics at Novogene Biotech Co., Ltd. The detection method has been optimized by the company.

As explained at the beginning, we did not perform targeted metabolomics. For LC-MS/MS quantification of differential bile acids: LC-MS/MS analyses were performed using an Agilent 1200 high performance LC system (Agilent Technologies, USA) coupled with a 4000Q TRAP triple quadrupole (QQQ) mass spectrometer (AB Sciex, USA). Turbo Ion Spray source was operated in the negative-ion multiple-reaction monitoring (MRM) mode. The MRM transitions of 5 bile acids were shown in Response Table 2. The ESI-MS parameters were the ion spray voltage, -4500 V; nebulizer gas (N2), 50 units; curtain gas (N2), 15 units; collision gas (N2), medium. The drying gas (N2) flow and temperature were 50 units and 500 ℃, respectively.

This information has been added to Method (page 29-32) in the revised manuscript.

Compound	m/z	Declustering potential (V)	Collision energy (V)
TCDCA	498.3/80.0	-180	-120
GCA	464.4/74.0	-115	-75
23-NorCA	393.4/329.4	-160	-47
T-α-MCA	514.4/80.0	-150	-87
CA	407.2 /343.4	-162	-45

Response Table 2. MRM transitions of 5 bile acids

f. Line 723: “Further validation via LC/MS/MS…”, what was the validation carried out?

Our response:

We are sorry for the confusion. In our study, we performed untargeted metabolomics at Novogene Biotech Co., Ltd, and found significant differences in some bile acids. To quantify the differential metabolites, we established LC-MS/MS methods to analyze specific bile acids in different strains of mice as shown in Fig. 3b-3e and Fig. S8d-8f. We confirmed the retention time of their peaks based on the standards. The absolute concentration of metabolites was calculated according to the standard curves, which were created using appropriate serial dilutions of the corresponding standards.

This information has been added to Method (page 29-32) in the revised manuscript.

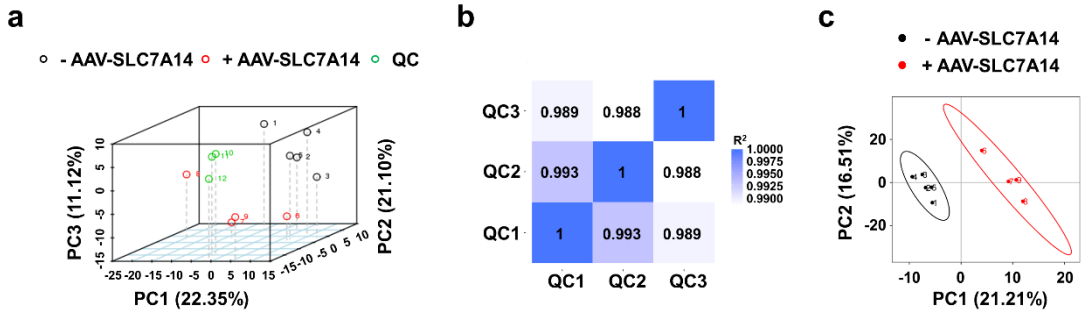
g. A principal component analysis (PCA) plot with all samples including QC samples is recommended in the supplement data.

Our response:

We appreciate it very much for the reviewer’s suggestion. In response, we performed the principal component analysis (PCA) plot with all samples including quality control (QC) and found that the QC samples clustered tightly together (Response Figure 1a), which indicating that the data was reliable. Additionally, QC data was performed by Pearson correlation analysis to obtain stable and accurate metabolome results (Gut. 2022 Jun;71(6):1203-1213). The Pearson correlation QC samples was high as shown in Response Figure 1b, indicating that the data quality was reliable and stable. Moreover, we used a multivariate statistical method, namely partial least squares discrimination analysis (PLS-DA) (Gut. 2022 Jun;71(6):1203-1213; Adv Sci (Weinh). 2024 Mar 1:e2309255), to assess the metabolic alterations among groups. We performed PLS-DA plot of serum metabolic profiling, which revealed overexpression of SLC7A14 (+ AAV-SLC7A14) groups could be distinguished from

the control (- AAV-SLC7A14) groups in Response Figure 1c.

This information has been added to Results (page 10) and Method (page 30-31) in the revised manuscript.



Response Figure 1. Untargeted metabolomics analysis

(a) Principal component analysis score plot of all samples containing QC samples.

(b) Pearson correlation between QC samples.

(c) Partial least squares-discriminant analysis of serum metabolic profiling.

Studies for a-c were conducted using serum of POMC Cre mice receiving AAVs expressing mCherry (- AAV-SLC7A14) or SLC7A14 (+ AAV-SLC7A14) in ARC. Principal components analysis (PCA) and Partial least squares discriminant analysis (PLS-DA) were performed at metaX.

REVIEWER COMMENTS

Reviewer #3 (Remarks to the Author):

1. It is suggested to thoroughly review the mass spectrometry, metabolomics, and chemometrics parts of the study, ensuring the accuracy of all provided information.

2. Considering the limitations of a small sample size, it is recommended to prioritize simpler methodologies such as t-tests/ANOVA, PCA, and heatmaps over the supervised classification method PLS-DA, which typically requires a larger sample size.

REVIEWER COMMENTS

Reviewer #3 (Remarks to the Author):

1. It is suggested to thoroughly review the mass spectrometry, metabolomics, and chemometrics parts of the study, ensuring the accuracy of all provided information.

Our response:

We appreciate it very much for the reviewer's suggestion. In response, we have checked all provided information of the mass spectrometry, metabolomics, and chemometrics parts of the study, and confirmed the accuracy of the information.

2. Considering the limitations of a small sample size, it is recommended to prioritize simpler methodologies such as t-tests/ANOVA, PCA, and heatmaps over the supervised classification method PLS-DA, which typically requires a larger sample size.

Our response:

We appreciate it very much for the reviewer's suggestion. In response, we have applied univariate analysis (t-test) to calculate the statistical significance (P-value) and added this results in Supplementary Data 1 of the revised manuscript (Response Table 1). Combining the PCA plot with all samples including quality control (QC) samples (Fig.S8a), we think the data quality of metabolomics is reliable and the taurochenodeoxycholic acid (TCDCA) is a differential metabolite in SPOE mice serum compared with controls. Furthermore, we agree with the reviewer's opinion that the PLS-DA method is not inappropriate. Therefore, we have removed PLS-DA from the revised manuscript.

This information has been added to Supplementary Data 1 in the revised manuscript.

Description:Data were obtained using untargeted metabolomics in control						
Compound_ID	Name	Formula	lecular.Wei	RT..min.	m.z	control1
Com_100_neg	LPC 20:3	C28 H52 N	605.3694	14.828	604.3622	1.93E+08
Com_101_neg	4-[2-(2-ox	C9 H17 N3	202.0953	1.129	201.0881	1.17E+08
Com_1015_neg	Hexadecan	C16 H30 O	286.2144	12.449	285.207	4923148
Com_1020_neg	OxPC (16:	C42 H80 N	833.5787	16.318	832.5716	1634332
Com_1034_neg	PEtOH (16	C39 H71 O	698.489	14.777	697.4818	20255972
Com_104_neg	LPE 20:4	C25 H44 N	501.2855	14.601	500.2782	3.79E+08
Com_1040_neg	FAHFA (18	C38 H66 O	586.4936	14.982	585.4865	6455405

Response Table 1 The raw data of untargeted metabolomics. Data were obtained using untargeted metabolomics in control mice (n = 5, labeled as control 1-5), SLC7A14 overexpressed mice (n = 4, labeled as SPOE 1-4) and quality control sample (n = 3, labeled as QC 1-3).

REVIEWER COMMENTS

Reviewer #3 (Remarks to the Author):

1. Figure 3.a. The authors mentioned that the metabolite set enrichment analysis (MSEA) was carried out using MetaboAnalyst 5.0, however, it was not clear what the input data used? It is suggested to provide additional information such as what was the input data (a list of mz/rt peaks or a list of annotated metabolites?), which module of MetaboAnalyst 5.0 was used, and details information for parameters to run the (MSEA).

2. It is suggested to provide additional information regarding the annotation of metabolites. Which ppm tolerance was used for the matching against a database? how the authors deal with the multiples matches (a queried mz may matched against different compounds)? For the most significands metabolites, it is also suggested to provide an identification level.

Reviewer #3 (Remarks to the Author):

1. The authors mentioned that the metabolite set enrichment analysis (MSEA) was carried out using MetaboAnalyst 5.0, however, it was not clear what the input data used? It is suggested to provide additional information such as what was the input data (a list of mz/rt peaks or a list of annotated metabolites?), which module of MetaboAnalyst 5.0 was used, and details information for parameters to run the (MSEA).

Our response:

Thanks for the reviewer's suggestion. In response, we used a list of differential metabolites names to perform metabolite set enrichment analysis (MSEA) on MetaboAnalyst 5.0, which was clarified in previous response letter. Regarding the input data, we provided a list of differential annotated metabolites in Response Table 1. Moreover, we used Enrichment Analysis module in MetaboAnalyst 5.0, and selected chemical structures (main-class) metabolite set library for MSEA. For more details information of MSEA, we have provided the R language code which provided by MetaboAnalyst 5.0 website in Response Data 1.

PA (16:0/18:2)
PC (18:1/22:6)
PC (16:0/20:4)
LPA 18:2
N-Glycolylneuraminic acid
Dulcitol
LPA 22:6
2-Hydroxycaproic acid
4-Oxoproline
Taurochenodeoxycholic acid
PC (16:1e/22:6)
8,15-Dihete
Sodium cholate
Piceatannol
Glycocholic acid
2-Methylbutylbeta-D-glucopyranoside
2-Isopropylmalate
Hexadecanedioic acid
PC (16:0/22:6)
23-Norcholic acid
L-Leucyl-L-alanine Hydrate
N-Acetylalanine
Tauro-alpha-Muricholic acid sodium salt

Response Table 1 a list of differential annotated metabolites

```
# PID of current job: 3217671
mSet<-InitDataObjects("conc", "msetora", FALSE)
cmpd.vec<-c("PA (16:0/18:2)","PC (18:1/22:6)","PC (16:0/20:4)","LPA 18:2","N-Glycolylneuraminic acid","Dulcitol","LPA 22:6","2-Hydroxycaproic acid","4-Oxoproline","Taurochenodeoxycholic acid","PC (16:1e/22:6)","8,15-Dihete","Sodium cholate","Piceatannol","Glycocholic acid","2-Methylbutyl beta-D-glucopyranoside","2-Isopropylmalate","Hexadecanedioic acid","PC (16:0/22:6)","23-Norcholic acid","L-Leucyl-L-alanine Hydrate","N-Acetylalanine","Tauro-alpha-Muricholic acid sodium salt")
mSet<-Setup.MapData(mSet, cmpd.vec);
mSet<-CrossReferencing(mSet, "name");
mSet<-CreateMappingResultTable(mSet)
mSet<-SetMetabolomeFilter(mSet, F);
mSet<-SetCurrentMsetLib(mSet, "main_class", 2);
mSet<-CalculateHyperScore(mSet)
mSet<-PlotORA(mSet, "ora_0_", "net", "png", 72, width=NA)
mSet<-PlotEnrichDotPlot(mSet, "ora", "ora_dot_0_", "png", 72, width=NA)
mSet<-PlotEnrichPieChart(mSet, "ora", "ora_pie_0_", "png", 72)
mSet<-CalculateHyperScore(mSet)
mSet<-PlotORA(mSet, "ora_1_", "net", "png", 72, width=NA)
mSet<-PlotEnrichDotPlot(mSet, "ora", "ora_dot_1_", "png", 72, width=NA)
mSet<-PlotEnrichPieChart(mSet, "ora", "ora_pie_1_", "png", 72)
mSet<-SaveTransformedData(mSet)
```

Response Data 1 The R language code for MSEA on MetaboAnalyst 5.0

This information has been added to Method (page 31) in the revised manuscript.

2. It is suggested to provide additional information regarding the annotation of metabolites. Which ppm tolerance was used for the matching against a database? how the authors deal with the multiples matches (a queried mz may matched against different compounds)? For the most significands metabolites, it is also suggested to provide an identification level.

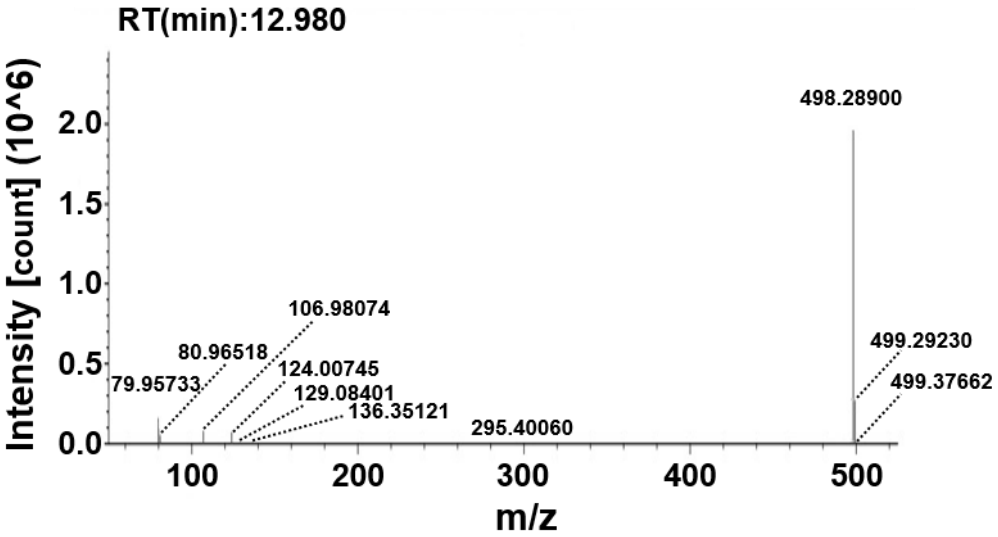
Our response:

Thanks for the reviewer's suggestion. In response, the untargeted metabolomics were performed at Novogene Biotech Co., Ltd, which has established extensive cooperative relationships with numerous academic institutions worldwide, and more than 22850 SCI articles have been coauthored or mentioned. The experimental process of untargeted metabolomics research is strictly controlled and standardized, and there are many articles on untargeted metabolomics were conducted in this company (Adv Sci (Weinh). 2024 May;11(18):e2309255, Adv Sci (Weinh). 2023 Mar;10(8):e2204826, Immunity. 2021 Aug 10;54(8):1728-1744.e7, Microbiome. 2020 Mar 10;8(1):32). There was 5ppm tolerance used for the matching against a database.

For multiples matches, the peak intensities were normalized to the total spectral

intensity. The normalized data was used to predict the molecular formula based on mass number deviation (ppm), additive ions, molecular ion peaks and fragment ions. And then peaks were matched with the mzCloud (https://www.mzcloud.org/), mzVault and MassList database to obtain the accurate qualitative and relative quantitative results. The database containing secondary spectra matches the actual secondary spectra with information such as fragment ions and collision energies of each metabolite in the database, achieving secondary identification of modern metabolites.

For the identification level of TCDCA, TCDCA was identified with a metabolomics standards initiative (MSI) level 2 identification (Response Figure 1).



Response Figure 1 The identification level of TCDCA

This information has been added to Results (page 10) and Method (page 30) in the revised manuscript.

REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

Several of my previous comments have been addressed. At this point, I have no additional comments regarding this manuscript.