nature portfolio

# Peer Review File

## Ngfr+ cholinergic projection from SI/nBM to mPFC selectively regulates temporal order recognition memory

Reviewer #1 (Remarks to the Author):

Mei et al reported that the Ngfr+ cholinergic neuron activity within the SI/nBM-mPFC circuit underlies the recent judgments during recognition memory tasks. The disruption of Ngfr function reduces the excitability of cholinergic neurons within the SI/nBM-mPFC circuit, but not in the MS-HP pathway. This disruption leads to a fiber photometry-detectable decrease in Ach release within the mPFC during object encounters. Importantly, NGFR-deficient mice display specific impairment in temporal order recognition memory, while their abilities to perform object and location recognition tasks remain intact. This observation supports the model that Ngfr+ cholinergic neuron activity within the SI/nBM-mPFC circuit selectively mediates temporal order recognition memory.

Inhibition of SI/nBM-mPFC cholinergic innervation with optogenetic and chemogenetic manipulation in ChAT-Cre mice results in severe temporal order recognition deficits, further emphasizing the importance of this circuit in this type of memory task. Physiological analysis indicates that the impairment of cholinergic activity leads to a depolarizing shift of GABAergic input to mPFC pyramidal neurons due to disturbed KCC2-mediated chloride gradients. Finally, restoration of Ach signaling by upregulation of KCC2 levels restores temporal order recognition deficits. The results are very interesting, but I still have several concerns.

1. The author adopted three different behavioral paradigms to investigate the role of NGFR in recognition memory. It was found that knocking out NGFR can specifically affect animals' temporal order recognition. Although the author also performed circuit-specific manipulations, which suggested that NGFR may be involved in temporal order recognition through SI/nBM-mPFC cholinergic projection, considering that the author used animals with global knockouts during the embryonic stage and that NGFR itself is a molecule that can affect nervous system development, the author should use rescue experiments or cell type-specific knockout animals to confirm whether the observed behavioral phenotypes are due to the effects of NGFR on cholinergic neuron function itself or because of the side effects of affecting nervous system development.

2. In Figure 4, the author used a fiber photometry system to analyze the dynamic changes of Ach in the mPFC during the temporal order recognition paradigm. This is a very interesting result, but unfortunately, the data analysis is too simple. During the object discrimination paradigm, animals gradually explore both novel and familiar objects, and this exploration process involves many behavioral details, such as approach and withdrawal. By combining the analysis of Ach dynamics and behavioral details of exploration, the author can obtain more specific information about the relationship between them, which would provide more detailed information about how Ach in the mPFC is involved in discrimination memory processes.

3. According to the results shown in Figure 4c, upon contacting an object, the mPFC releases Ach, and knocking out Ngfr can completely inhibit this response. Based on the behavioral phenotypes of the three paradigms, knocking out NGFR specifically affects temporal order recognition without affecting the other two paradigms. Does this suggest that the mPFC

is not involved in the other two behaviors? This needs to be further tested by the authors. To strengthen the conclusion, the author should also examine the dynamic changes of Ach during the other two paradigms.

4. The results in Figure 6 are very impressive, and the author clearly demonstrates that GABAergic neurons in Ngfr-/- mice undergo a depolarizing shift in their input to the mPFC. The author suggests that this depolarizing shift leads to behavioral deficits in Ngfr-/- mice. In Figure 7, the author treats animals with chronic Nicotine and is able to rescue the behavioral deficits. The author should also test whether this treatment can rescue the previous depolarizing shift, as this would complete the entire logical chain.

REVIEWER COMMENTS

Reviewer #2 (Remarks to the Author):

General comments:

The present study reported that NGFR signaling plays an important role in the excitability of SI/nBM cholinergic neurons and temporal order recognition memory. The behavioral role of SI/nBM cholinergic neurons projecting to the mPFC was confirmed by chemogenetic and optogenetic inhibition experiments. This signaling is also involved in depolarizing shift of GABAergic inputs to mPFC pyramidal neurons through the control of KCC2 expression level.

These data provide new information on the function of NGFR signaling in recognition memory and electrophysiological response of GABA inputs to mPFC neurons in conjunction with cholinergic function.

However, there are some major concerns to explain their results correctly based on the experimental data as follows.

1. The authors described that Ngfr-positive cholinergic neurons projecting from the SI/nBM to the mPFC selectively regulate temporal order recognition memory. This study indicated the importance of NGFR signaling in temporal order recognition memory by using the knockout mice, but SI/nBM neurons may be also involved in novel object recognition memory as reported in other studies (Okada et al., 2015). Therefore, it is unclear whether SI/nBM neurons projecting to the mPFC selectively contribute to the temporal order recognition. Chemogenetic or optogenetic inhibition experiments may be needed to test the novel object recognition.

The mPFC receives the inputs not only from the SI/nBM but also from the MS/vDDB. Based on the chemogenetic or optogenetic experiment, it is difficult to conclude that the SI/nBM-mPFC cholinergic activity selectively contributes to temporal recognition memory.

In addition, previous studies report changes in cholinergic cell number in NGFR deficient mice (Greferath et al., 2000; Naumann et al., 2002; Peterson et al., 1997, 1999). These changes may affect the recognition memory in the knockout mice. Did you check the cell number of cholinergic neurons in the SI/nBM in the mice used herein? Both of changed cholinergic cell number and NGFR deficiency may cause the behavioral phenotype in the knockout mice.

2. The authors mentioned that Ngfr-positive cholinergic neurons regulate temporal order recognition memory through controlling GABAergic transmission. This study demonstrated the impaired depolarizing shift of GABAergic inputs to mPFC pyramidal neurons in the knockout mice by using electrophysiological recordings. However, the role of GABAergic regulation of pyramidal neurons in recognition memory has not been tested. Therefore, there is no direct evidence that GABAergic regulation of pyramidal neurons controls the recognition memory.

3. ACh release was increased at the time window around object contacts in all phases (sample and test phases) of the temporal order recognition task in the control mice, whereas the release level was significantly decreased in all phases in the knockout mice (Figure 4). ACh release seems to be increased in association with object contacts. How do you explain the mechanism of recognition memory by increased ACh release? In other recognition memory tasks, the condition at the sample phase is the same as one in the temporal order recognition task. Thus, the release response would be similar between different recognition memory tasks. The data showing the normal behavior of knockouts on other recognition memory tasks suggest that Ach release at the sample phase is not important for recognition memory. The authors should measure ACh release at the test phase of other recognition memory tasks. The release response at the test phase may be different among the recognition memory tasks with or without the effects of the knockout.

4. Statistical data (F values) are not shown throughout the manuscript.

Minor comments:

p. 5, line 38:

"saporin" should be expressed as "IgG 192 saporin".

p.7. 1ine 21

In Supplementary Fig. 3a, the number of cells expressing hM4DiR in the SI/nBM appears to be low. Show the magnified views of this area.

p.10, line 5:

KCC2 level is largely different between the knockouts in Figure 6, panel h (~0.5) and the controls in Figure 7, panel a (~1.0).

p.11, line 28:

“Descarries, 1997”. Citation procedure is incorrect.

p.12, lines 22-23:

Which results show the inhibitory synaptic numbers of pyramidal neurons?

p.13, line 7:

What kinds of growth factors or morphogens are involved in the retrograde signals or target-specific cues?“cholineric” should be “cholinergic”.

p.16, lines 11-14:

What is the genome titer of each AAV vector?

p.16, line 30:

Show the dilution of primary antibody. For secondary antibody, show the maker and dilution.

p.17, lines 13-15:

Show the recovery period after the surgery and the timing of CNO treatment.

p.26 line 29:

Show the scale bar for Figure 4, panel b, and the size in its legend.

Reviewer #3 (Remarks to the Author):

The study by Mei et al. uses multidisciplinary approaches including behavioral tests, molecular assays, imaging (optogenetics, fiberphotometry), pharmacology and electrophysiology to investigate the role of Ngfr+ cholinergic neurons, localized in the SI/nBM and projecting to mPFC, in recognition memory. Results show that Ngfr signaling controls SI/nBM cholinergic neurons excitability, ACh levels and GABA polarity in the mPFC targeted neurons. Furthermore, the absence of Ngfr signaling impairs temporal order but not object recognition or object location memory.

Overall the manuscript is well written and the data nicely presented. The study is of great interest for the field but some weaknesses arise. Major and minor points are listed below.

Major points:

1) The majority of results have been obtained from constitutive Ngfr knockout (Ngfr-/-) mice. These data do not pin down the reason for defects observed in the adult stage to the immature stage, the adult one or both and do not exclude possible compensatory mechanisms (for instance, the level of Trka expression). Extra experiments might be needed to clarify this point, such as deleting/knocking down Ngfr in cholinergic neurons specifically in the adult stage and then check the potential changes on neuron excitability, GABA polarity and behaviors.

2) It has been shown that p75NTR is expressed in glial cells as well, thus rescue experiments with Ngfr selective replacement in SI/nBM cholinergic neurons would exclude possible indirect effects of Ngfr deletion.

3) p75NTR activation mediates apoptotic signaling. Although it is a matter of debate, it would be important to show whether the number of SI/nBM cholinergic neurons and their projections in the mPFC are affected or not in the Ngfr-/- mice used in this study. These data could support the reduction of ACh levels observed in the mPFC of Ngfr-/- mice.

Minor points:

1) please justify the use of male mice and specify whether only males where used in the different experimental sets (behavior, molecular biology and electrophysiology).

3) add the mouse strain stock code in the method section.

4) please clarify whether the experiments were performed in blind condition (genotype and/or treatment).

5) add the viral titers in the method section.

6) add a section about the analysis of electrophysiological data sets in the method section.

7) add the concentration of antibodies and DAPI.

8) add traces in supplementary figure 4I.

9) It would be important to discuss the effect of carbachol on the firing of layer V pyramidal cells in Ngfr-/- as compared to control mice.

10) Figure 7: saccharin solution and saccharin solution + nicotine should be administered also to Ngfr+/+ mice.

REVIEWER COMMENTS

**Reviewer #1**

*Mei et al reported that the Ngfr+ cholinergic neuron activity within the SI/nBM-mPFC circuit underlies the recent judgments during recognition memory tasks. The disruption of Ngfr function reduces the excitability of cholinergic neurons within the SI/nBM-mPFC circuit, but not in the MS-HP pathway. This disruption leads to a fiber photometry-detectable decrease in Ach release within the mPFC during object encounters. Importantly, NGFR-deficient mice display specific impairment in temporal order recognition memory, while their abilities to perform object and location recognition tasks remain intact. This observation supports the model that Ngfr+ cholinergic neuron activity within the SI/nBM-mPFC circuit selectively mediates temporal order recognition memory.*

*Inhibition of SI/nBM-mPFC cholinergic innervation with optogenetic and chemogenetic manipulation in ChAT-Cre mice results in severe temporal order recognition deficits, further emphasizing the importance of this circuit in this type of memory task. Physiological analysis indicates that the impairment of cholinergic activity leads to a depolarizing shift of GABAergic input to mPFC pyramidal neurons due to disturbed KCC2-mediated chloride gradients. Finally, restoration of Ach signaling by upregulation of KCC2 levels restores temporal order recognition deficits. The results are very interesting, but I still have several concerns.*

We appreciated the reviewer for his/her critical and constructive comments, which helped us to improve our manuscript.

*1. The author adopted three different behavioral paradigms to investigate the role of NGFR in recognition memory. It was found that knocking out NGFR can specifically affect animals' temporal order recognition. Although the author also performed circuit-specific manipulations, which suggested that NGFR may be involved in temporal order recognition through SI/nBM-mPFC cholinergic projection, considering that the author used animals with global knockouts during the embryonic stage and that NGFR itself is a molecule that can affect nervous system development, the author should use rescue experiments **or** cell type-specific knockout animals to confirm whether the observed behavioral phenotypes are due to the effects of NGFR on cholinergic neuron function itself or because of the side effects of affecting nervous system development.*

**[Response]** The reviewer raised an important question: whether temporal order recognition deficits in *Ngfr*$^{-/-}$ mice were attributable to development compensatory effects. To address this question, we have performed both rescue experiments in adult *Ngfr*$^{-/-}$ mice and cholinergic-specific knockdown of *Ngfr* experiments in adult wild-type (WT) mice. Specifically, we bilaterally injected *Ngfr*-expressing AAV9 virus under the control of cholinergic specific promoter into the SI/nBM of adult *Ngfr*$^{-/-}$ mice at ages of 2 to 5 months. Immunofluorescence staining revealed selective re-expression of *Ngfr* in the *Ngfr*$^{-/-}$ SI/nBM cholinergic neurons (new **Supplementary Fig. 3a**, bottom row). Behavioral tests were performed 4 weeks after virus injection. Notably, *Ngfr* expression rescued temporal order recognition deficits in the *Ngfr*$^{-/-}$ mice, without affecting novel object recognition and object location recognition (new **Supplementary Fig. 3b, c, d**). Moreover, we bilaterally injected AAV9-ChAT mini TK-miR30shRNA (*Ngfr*), which would knock down *Ngfr* expression through antisense shRNA, or the control virus into the SI/nBM of adult WT mice at ages of 2 to 3 months. Immunofluorescence staining revealed a marked reduction in *Ngfr* expression in the wild-type SI/nBM cholinergic neurons (new **Supplementary Fig. 3e**). Consistently, reduction in *Ngfr*

expression significantly impaired temporal order recognition memory, leaving intact the novel object recognition and the object location recognition (new **Supplementary Fig. 3f, g, h**). Taken together, these data provided solid evidence that *Ngfr* expression in the SI/nBM controls temporal order recognition by regulating the electrophysiological function of the cholinergic neurons itself in the adult, rather than affecting nervous system development in general.

*2. In Figure 4, the author used a fiber photometry system to analyze the dynamic changes of Ach in the mPFC during the temporal order recognition paradigm. This is a very interesting result, but unfortunately, the data analysis is too simple. During the object discrimination paradigm, animals gradually explore both novel and familiar objects, and this exploration process involves many behavioral details, such as approach and withdrawal. By combining the analysis of Ach dynamics and behavioral details of exploration, the author can obtain more specific information about the relationship between them, which would provide more detailed information about how Ach in the mPFC is involved in discrimination memory processes.*

**[Response]** The reviewer raised an important question: how ACh release in the mPFC is involved in the recency judgments in recognition memory. We have now designed new experiments to address this question. First, we monitored the ACh dynamics in the mPFC along with detailed behavioral changes during temporal order recognition. We found that ACh signals rose right before mice encountered the object (time 0) and declined rapidly when they withdrew from exploring the objects. The data are shown in the **Fig. 4c-d** and **Supplementary video 1**. These results suggest ACh signal may function as an initiator for object recognition. Second, we determined whether ACh release is involved in object discrimination and recency judgements during recognition tasks (new **Fig. 4a**). We measured ACh release during objects encounters (time 0) in the sample phases and test phase in WT mice. There was a rise in ACh release during object encounter, but no significant difference in ACh release when the mice explored the two identical objects, either in the sample phase 1 or sample phase 2 (new **Fig. 4e**, left and middle). Remarkably, in the test phase, ACh signals were significantly higher when the mice explored the earlier object from sample phase 1 than that when they explored the later object from sample phase 2 (new **Fig. 4e**, right). These data suggest that ACh signal plays a crucial role in discriminating earlier versus later objects. Taken together, these results suggest an intriguing note that a rise of ACh not only predicts object encounters but also determines recency judgements in recognition memory.

*3. According to the results shown in Figure 4c, upon contacting an object, the mPFC releases Ach, and knocking out Ngfr can completely inhibit this response. Based on the behavioral phenotypes of the three paradigms, knocking out NGFR specifically affects temporal order recognition without affecting the other two paradigms. Does this suggest that the mPFC is not involved in the other two behaviors? This needs to be further tested by the authors. To strengthen the conclusion, the author should also examine the dynamic changes of Ach during the other two paradigms.*

**[Response]** The reviewer raised a series of questions regarding the differential effects of mPFC ACh release in three types of recognition memory: temporal order recognition, novel object recognition and object location recognition. The following efforts were made to address these questions. (1) Previous lesion studies suggest that mPFC is specifically involved in temporal order recognition, but not in novel object and object location recognition [1]. (2) We have now systematically examined dynamic changes of ACh release in the mPFC during novel object and object location recognition. In novel object recognition test (new **Supplementary Fig. 4a**), ACh release in mPFC only occurred in the sample phase (new **Supplementary Fig. 4b, c, d, left**),

with no detectable ACh signals in the test phase (new **Supplementary Fig. 4b, c, d, right**). Likewise, in object location recognition test (new **Supplementary Fig. 4e**), the rise of ACh signals in mPFC were seen only in the sample phase but not in the test phase (new **Supplementary Fig. 4f, g, h**). These findings suggest that ACh signals in the mPFC may be associated with object encounter (memory encoding), but not involved in the recognition of novel object or its specific location, i.e. not in the recognition memory retrieval process per se. (3) Although *Ngfr* knockout significantly reduced ACh signals in the encoding stage of novel object and object location recognition (new **Supplementary Fig. 4b-d, 4f-h**), the behavioral outcomes of these two types of recognition remained intact (**Fig. 3f-3k**). We speculate that defected ACh release in the mPFC during the encoding stage of recognition may not be sufficient to interrupt discrimination of object familiarity or spatial recognition. Indeed, lesions in the mPFC per se do not affect novel object and object location recognition[1]. Collectively, our findings highlight the pivotal role of the mPFC and *Ngfr*-dependent ACh release in mPFC in temporal order recognition, but not in novel object and object location recognition.

*4. The results in Figure 6 are very impressive, and the author clearly demonstrates that GABAergic neurons in Ngfr-/- mice undergo a depolarizing shift in their input to the mPFC. The author suggests that this depolarizing shift leads to behavioral deficits in Ngfr-/- mice. In Figure 7, the author treats animals with chronic Nicotine and is able to rescue the behavioral deficits. The author should also test whether this treatment can rescue the previous depolarizing shift, as this would complete the entire logical chain*

**[Response]** We appreciated the reviewer's suggestion, and have now demonstrated that chronic nicotine treatment rescued depolarizing shift of the reversal potential of $E_{IPSCs}$ seen in *Ngfr*$^{-/-}$ mPFC. Specifically, perforated-patch recordings of mPFC layer V pyramidal neurons were made from *Ngfr*$^{-/-}$ mice treated with nicotine in the drinking water at the dose of 650 μg/ml over 4-week period. We found a dramatic reversal (rescue) of the depolarizing-shifted $E_{IPSCs}$, from -79.23±3.79mV to -109.5±6.41 mV in the *Ngfr*$^{-/-}$ mPFC, closer to that from WT mPFC (new **Fig. 7b, c, d, e**). Thus, we provided direct evidence that ACh receptor agonist nicotine regulates GABAergic driving force, which plays a key role in temporal order recognition memory.

**Reviewer #2 (Remarks to the Author):**

*General comments:*
*The present study reported that NGFR signaling plays an important role in the excitability of SI/nBM cholinergic neurons and temporal order recognition memory. The behavioral role of SI/nBM cholinergic neurons projecting to the mPFC was confirmed by chemogenetic and optogenetic inhibition experiments. This signaling is also involved in depolarizing shift of GABAergic inputs to mPFC pyramidal neurons through the control of KCC2 expression level. These data provide new information on the function of NGFR signaling in recognition memory and electrophysiological response of GABA inputs to mPFC neurons in conjunction with cholinergic function.*

*However, there are some major concerns to explain their results correctly based on the experimental data as follows.*

*1. The authors described that Ngfr-positive cholinergic neurons projecting from the SI/nBM to the mPFC selectively regulate temporal order recognition memory. This study indicated the importance of NGFR signaling in temporal order recognition memory by using the knockout mice, but SI/nBM neurons may be also involved in novel object recognition memory as reported in other studies (Okada et al., 2015). Therefore, it is unclear whether SI/nBM neurons projecting to the mPFC selectively contribute to the temporal order recognition. Chemogenetic or optogenetic inhibition experiments may be needed to test the novel object recognition.*
**[Response]** We appreciated the reviewer's comment, and have now shown that the SI/nBM-mPFC cholinergic circuit selectively regulates temporal order but not novel object recognition memory. Specifically, we altered the SI/nBM-mPFC circuit using AAV-retro virus delivering hM4D(Gi) in ChAT-Cre mice. Bilateral injection of the AAV-retro virus into the mPFC enabled its retrograde transportation to SI/nBM, and specifically manipulated the ChAT-expressing and mPFC projecting SI/nBM cholinergic neurons (**Fig. 5a and** new **Supplementary Fig. 5a**). Chemogenetic inhibition of the SI/nBM-mPFC circuit significantly impaired temporal order recognition (**Fig. 5b**), but NOT the novel object recognition memory (new **Supplementary Fig. 5b**). This observation is consistent with previous studies showing that cortical lesions in the perirhinal cortex (PRH), but not mPFC, lead to novel object recognition memory deficits [1]. Thus, while SI/nBM cholinergic neurons are involved in both forms of recognition memory, the SI/nBM-mPFC circuit controls temporal order whereas SI/nBM-PRH projection controls novel object recognition.

*The mPFC receives the inputs not only from the SI/nBM but also from the MS/vDDB. Based on the chemogenetic or optogenetic experiment, it is difficult to conclude that the SI/nBM-mPFC cholinergic activity selectively contributes to temporal recognition memory.*
**[Response]** Thanks for the suggestion. To address this question, we have specifically inactivated MS/vDB cholinergic neurons by chemogenetic inhibition and showed that MS/vDB cholinergic activity is not involved in temporal order recognition. Specifically, AAV virus carrying hM4D(Gi) was injected into the MS/vDB of ChAT-cre mice to achieve specific inactivation of the cholinergic subpopulation (new **Supplementary Fig. 5c**). Interestingly, chemogenetic inhibition of the MS/vDB cholinergic neurons did not affect temporal order recognition (new **Supplementary Fig. 5d**). Taken together, our data suggest that mPFC cholinergic projections from SI/nBM but not those in MS/vDB are critical for temporal order recognition memory.

*In addition, previous studies report changes in cholinergic cell number in NGFR deficient mice (Greferath et al., 2000; Naumann et al., 2002; Peterson et al., 1997, 1999). These changes may affect the recognition memory in the knockout mice. Did you check the cell number of cholinergic neurons in the SI/nBM in the mice used herein? Both of changed cholinergic cell number and NGFR deficiency may cause the behavioral phenotype in the knockout mice.*

**[Response]** Thanks for the suggestion. Whether there is a change in the cholinergic cell number in the *Ngfr*$^{-/-}$ mice is a matter of debate. It should be noted that previous studies reported either increased or decreased cell number of cholinergic neurons in the *Ngfr*$^{-/-}$ MS/vDB[2-10]. Whether loss of NGFR affects SI/nBM was not fully studied. We have now shown that there was no significant difference in the cell number of cholinergic neurons in the SI/nBM between the adult *Ngfr*$^{+/+}$ and *Ngfr*$^{-/-}$ mice at the stage we used the animals (3 to 7 months) (new **Supplementary Fig. 2c, d**). *Ngfr* may exert differential effects on various cholinergic subpopulations, and in SI/nBM the primary function of *Ngfr* is to regulate cholinergic excitability and ACh release, rather than controlling cell survival. We thus argue that it is the *Ngfr*-dependent cholinergic activity but not the cholinergic cell number that determines the temporal order recognition memory in our model system.

*2. The authors mentioned that Ngfr-positive cholinergic neurons regulate temporal order recognition memory through controlling GABAergic transmission. This study demonstrated the impaired depolarizing shift of GABAergic inputs to mPFC pyramidal neurons in the knockout mice by using electrophysiological recordings. However, the role of GABAergic regulation of pyramidal neurons in recognition memory has not been tested. Therefore, there is no direct evidence that GABAergic regulation of pyramidal neurons controls the recognition memory.*

**[Response]** We appreciated the reviewer for this question, which helped us to improve the entire logic chain in the revised manuscript. We have shown that a reduction in KCC2 expression resulted in a depolarizing shift of GABAergic inputs to mPFC pyramid neurons in the *Ngfr*$^{-/-}$ mice, leading to a selective impairment in temporal order recognition (**Fig. 6**). Further, we bilaterally injected furosemide through cannula, a well-established KCC2 inhibitor, into the mPFC of adult wild-type (WT) mice at ages of 3 months, and performed three types of recognition memory tests. Remarkably, mPFC delivery of furosemide specifically attenuated temporal order recognition (new **Supplementary Fig. 7d**), but NOT novel object recognition or object location memory (new **Supplementary Fig. 7e, f**). Our findings provide direct evidence that KCC2-mediated GABAergic driving force on mPFC pyramidal neurons controls recognition memory and strengthens the specific role of mPFC in the temporal order recognition, but not in the novel object and object location recognition.

*3. ACh release was increased at the time window around object contacts in all phases (sample and test phases) of the temporal order recognition task in the control mice, whereas the release level was significantly decreased in all phases in the knockout mice (Figure 4). ACh release seems to be increased in association with object contacts. How do you explain the mechanism of recognition memory by increased ACh release? In other recognition memory tasks, the condition at the sample phase is the same as one in the temporal order recognition task. Thus, the release response would be similar between different recognition memory tasks. The data showing the normal behavior of knockouts on other recognition memory tasks suggest that Ach release at the sample phase is not important for recognition memory. The authors should measure ACh release at the test phase of other recognition memory tasks. The release response at the test phase may be different among the recognition memory tasks with or without the effects of the knockout.*

**[Response]** The reviewer raised a series of questions regarding the physiological significance of increased ACh release in the mPFC and the underlying mechanism of recognition memory. The following efforts were made to address these questions.
(1) We have provided a video to show that ACh signal surges shortly prior to object encounter and rapidly declines when the mice withdrawal from exploration, suggesting that the ACh signal may function as an initiator for object recognition (new **Supplementary video 1**).
(2) We have quantitatively compared the strength of ACh release when the mice explore the earlier acquaintance (green circles) or the later acquaintance (purple squares) objects (new **Fig. 4a**). The ACh signals in the sample phase 1 and 2, in which the mice explored two identical objects, were equal (new **Fig. 4e**, left and middle). Remarkably, during the test phase, the ACh release was significantly higher when the mice explored the objects that they had encountered earlier, compared to those they had encountered later (new **Fig. 4e**, right). These data suggest that ACh signal not only predicts object encounter, but also directly determines recency judgments of objects.
(3) We systematically examined dynamic changes of ACh release in the mPFC during novel object and object location recognition. In contrast to temporal order recognition, ACh release in the mPFC of WT mice during novel object recognition was detected only in the sample phase (new **Supplementary Fig.4a, b, c, d**, left), but not in the test phase (new **Supplementary Fig.4a, b, c, d**, right). Likewise, in the object location recognition, elevated ACh signals were seen only in the sample phase (new **Supplementary Fig.4e, f, g, h**, left) but not in the test phase (new **Supplementary Fig.4e, f, g, h**, left). These data suggest that ACh signals in the mPFC are not involved in the memory retrieval of novel object and object location recognition.
(4) Although *Ngfr*$^{-/-}$ mice exhibited reduced ACh signals in the sample phase of novel object and object location recognition (new **Supplementary Fig.4b-d**, **f-h**), the behavioral outcomes of these two types of recognition remained intact. We speculate that defected ACh release during the encoding stage of recognition may not be sufficient to interrupt discrimination of object familiarity or spatial recognition. Indeed, lesions in the mPFC per se do not affect novel object and object location recognition [1].Taken together, our findings highlight the pivotal role of the mPFC and *Ngfr*-dependent ACh release in the mPFC in temporal order recognition memory, but not in novel object and object location recognition.

4. Statistical data (F values) are not shown throughout the manuscript.
**[Response]** We appreciated the reviewer for this question and have strengthened statistical data with F values in **Fig.2c, 2e, 3e** and **Supplementary Fig. 6g, 6h, 6j**.

*Minor comments:*
*p. 5, line 38:*
*"saporin" should be expressed as "IgG 192 saporin".*
**[Response]** We have now corrected it as "IgG 192 saporin".

*p.7. 1ine 21*
*In Supplementary Fig. 3a, the number of cells expressing hM4DiR in the SI/nBM appears to be low. Show the magnified views of this area.*
**[Response]** We have revised the immunofluorescence images in the revised manuscript (new **Supplementary Fig.5a)**

*p.10, line 5:*

*KCC2 level is largely different between the knockouts in Figure 6, panel h (~0.5) and the controls in Figure 7, panel a (~1.0).*

**[Response]** In the **Fig. 7a** in the previous manuscript, we normalized the control treatment of *Ngfr*$^{-/-}$ mice as "1" and presented KCC2 levels of nicotine treatment group as fraction of the control. According to the review's suggestion, we normalized nicotine treatment as "1" and calculated the KCC2 levels of controls as fraction of the nicotine treatment (new **Fig. 7a**).

*p.11, line 28:*
*"Descarries, 1997". Citation procedure is incorrect.*

**[Response]** We appreciated the reviewer for pointing out the apparent error, and have now corrected the citation procedure in the revised manuscript.

*p.12, lines 22-23:*
*Which results show the inhibitory synaptic numbers of pyramidal neurons?*

**[Response]** In **Supplementary Fig. 6b** in the revised manuscript, intact mIPSCs amplitude reflects no significant changes in inhibitory synaptic numbers of pyramidal neurons.

*p.13, line 7:*
*What kinds of growth factors or morphogens are involved in the retrograde signals or target-specific cues? "cholineric" should be "cholinergic".*

**[Response]** We have specified the growth factors (NGF, BDNF, CNTF, NT3) and morphogens (SHH, RA, FGF8, BMP9) that are involved in the retrograde signals and target-specific cues in the revised manuscript. We have corrected the typo "cholinergic".

*p.16, lines 11-14:*
*What is the genome titer of each AAV vector?*

**[Response]** We have notified the titer of each AAV vector in the revised manuscript.

*p.16, line 30:*
*Show the dilution of primary antibody. For secondary antibody, show the maker and dilution.*

**[Response]** We have specified the dilution and maker of primary and secondary antibody in the Methods section.

*p.17, lines 13-15:*
*Show the recovery period after the surgery and the timing of CNO treatment.*

**[Response]** We have specified a recovery period of 4 weeks after the virus injection in the Methods section. Mice were injected with 1 mg/kg Clozapine N-oxide (CNO) (s.c., 1 mg/ml in 0.9% saline) 1 hour before behavioral tests.

*p.26 line 29:*
*Show the scale bar for Figure 4, panel b, and the size in its legend.*

**[Response]** We have shown the scale bar for new **Fig. 4b** and the scale bar in the revised Figure Legend.

**Reviewer #3 (Remarks to the Author):**

*The study by Mei et al. uses multidisciplinary approaches including behavioral tests, molecular assays, imaging (optogenetics, fiberphotometry), pharmacology and electrophysiology to investigate the role of Ngfr+ cholinergic neurons, localized in the SI/nBM and projecting to mPFC, in recognition memory. Results show that Ngfr signaling controls SI/nBM cholinergic neurons excitability, ACh levels and GABA polarity in the mPFC targeted neurons. Furthermore, the absence of Ngfr signaling impairs temporal order but not object recognition or object location memory.*

*Overall, the manuscript is well written and the data nicely presented. The study is of great interest for the field but some weaknesses arise. Major and minor points are listed below.*

We appreciated the reviewer for his/her positive comments, which helped us to improve our manuscript.

*Major points:*

*1.The majority of results have been obtained from constitutive Ngfr knockout (Ngfr-/-) mice. These data do not pin down the reason for defects observed in the adult stage to the immature stage, the adult one or both and do not exclude possible compensatory mechanisms (for instance, the level of Trka expression). Extra experiments might be needed to clarify this point, such as deleting/knocking down Ngfr in cholinergic neurons specifically in the adult stage and then check the potential changes on neuron excitability, GABA polarity and behaviors.*

**[Response]** The reviewer raised an important question: whether the observed phenotypes in the adult *Ngfr*$^{-/-}$ mice were attributable to development compensatory effects. We have applied different strategies to address this question. First, we performed cholinergic-specific knockdown of *Ngfr* in the adult wild-type (WT) mice. Specifically, we bilaterally injected AAV-ChAT mini TK-miR30shRNA (*Ngfr*), which would knock down *Ngfr* expression through antisense shRNA, or the control virus into the SI/nBM of WT mice at ages of 2 to 3 months old. Immunofluorescence revealed a marked reduction in *Ngfr* expression in the WT SI/nBM cholinergic neurons (new **Supplementary Fig. 3e**). A reduction in *Ngfr* expression in the SI/nBM cholinergic neurons significantly impaired temporal order recognition (new **Supplementary Fig. 3f**), but not novel object recognition and object location recognition (new **Supplementary Fig. 3g, h**). These data indicate that *Ngfr* expression in the adult, rather than during development, is important for temporal order recognition memory. Second, we strengthened the conclusion that *Ngfr*-mediated regulation of cholinergic activity in the adult stage is essential for temporal order recognition memory. We have provided evidence that chronic treatment with nicotine in the adult *Ngfr*$^{-/-}$ mice rescued the deficit in temporal order recognition (new **Fig. 7f, g**). Moreover, we demonstrated that chronic treatment with nicotine restored GABAergic polarity in the adult *Ngfr*$^{-/-}$ mice (new **Fig. 7b, c**, **d, e**). Specifically, perforated-patch recordings of mPFC layer V pyramidal neurons were made from 2-month-old *Ngfr*$^{-/-}$ mice treated with nicotine in the drinking water at the dose of 650 μg/ml over 4-week period. We found a dramatic depolarizing-shifted reversal potential of $E_{IPSCs}$ from -79.23±3.79 mV to -109.5±6.41 mV, closer to that from WT mice. These results suggest that nicotine treatment restored GABAergic driving force on the mPFC pyramidal neurons, which underlies mechanism of improved temporal order recognition. Taken together, these data suggest that *Ngfr* expression in the adult stage and its regulation of cholinergic activity is essential for temporal order recognition.

One caveat of our experiment is that we were not able to record cholinergic neuron excitability in this knocking down mice system. The AAV-mediated expression of *Ngfr* shRNA under the ChAT mini TK promoter may be leaky, and there might be some non-specific expression in non-cholinergic neurons (new **Supplementary Fig.3e**). Thus, we were unable to specifically recognize cholinergic neurons with fluorescence and record their excitability in this setup.

*2. It has been shown that p75NTR is expressed in glial cells as well, thus rescue experiments with Ngfr selective replacement in SI/nBM cholinergic neurons would exclude possible indirect effects of Ngfr deletion.*

**[Response]** We have performed the rescue experiments with *Ngfr* selective replacement in the *Ngfr*$^{-/-}$ SI/nBM cholinergic neurons. Specifically, we injected *Ngfr*-expressing AAV9 under the control of cholinergic specific promoter into the SI/nBM of adult *Ngfr*$^{-/-}$ mice at ages of 2 to 5 months. Immunofluorescence staining revealed significant NGFR immunoreactivity in SI/nBM but not in other regions, indicating re-expression of NGFR in the *Ngfr*$^{-/-}$ mice (new **Supplementary Fig. 3a**, bottom row). Behavioral tests were performed 4 weeks after *Ngfr*-virus injection. Notably, *Ngfr* expression rescued the deficit in temporal order recognition memory (new **Supplementary Fig. 3b**), with little effect on novel object recognition and object location recognition (new **Supplementary Fig. 3c, d**). These data strengthened the physiological significance of *Ngfr* on cholinergic neuron function and temporal order recognition memory.

*3. p75NTR activation mediates apoptotic signaling. Although it is a matter of debate, it would be important to show whether the number of SI/nBM cholinergic neurons and their projections in the mPFC are affected or not in the Ngfr-/- mice used in this study. These data could support the reduction of ACh levels observed in the mPFC of Ngfr-/- mice.*

**[Response]** We appreciated the reviewer for this important question, and have now shown that there was no significant difference in the number of cholinergic neurons in the SI/nBM between the adult *Ngfr*$^{+/+}$ and *Ngfr*$^{-/-}$ mice (new **Supplementary Fig. 2c, d**). It should be noted that previous studies reported either increased or decreased cell number of cholinergic neurons in the *Ngfr*$^{-/-}$ MS/vDB[2-10]. Whether loss of NGFR affects SI/nBM was not fully studied. We now show that SI/nBM exhibited similar number of ChAT$^{+}$ cells in *Ngfr*$^{+/+}$ and *Ngfr*$^{-/-}$ mice. Thus, *Ngfr* may exert differential effects on various cholinergic subpopulations, and in SI/nBM the primary function of *Ngfr* is to regulate cholinergic excitability and ACh release, rather than controlling cell survival. We thus argue that it is the NGFR-mediated cholinergic activity but not the cholinergic cell number that determines the mPFC ACh levels in our model system.

*Minor points:*

*1. please justify the use of male mice and specify whether only males where used in the different experimental sets (behavior, molecular biology and electrophysiology).*

**[Response]** The behavioral and electrophysiological experiments were performed in male mice, attributable to prelusion of cyclic hormone effects of female mice. The molecular biology experiments were performed in both male and female mice. We have notified the gender of mice in the Method section.

*2. add the mouse strain stock code in the method section.*

**[Response]** We have added the mouse strain stock code 002213 in the Methods section.

*3. please clarify whether the experiments were performed in blind condition (genotype and/or treatment).*

**[Response]** We have clarified that experiments and data analyses were conducted by experimenters that were blinded to the genotype and experimental groups in the Methods section.

*4. add the viral titers in the method section.*
**[Response]** We have added viral titers in the Methods section.

*5. add a section about the analysis of electrophysiological data sets in the method section.*
**[Response]** We have added a section about the analysis of electrophysiological data sets in the Methods section.

*6. add the concentration of antibodies and DAPI.*
**[Response]** We have specified the dilution of antibodies and DAPI in the Methods section.

*7. add traces in supplementary figure 4I.*
**[Response]** We have added representative traces in new **Supplementary Fig. 6i**.

*8. It would be important to discuss the effect of carbachol on the firing of layer V pyramidal cells in Ngfr-/- as compared to control mice.*
**[Response]** We have used both carbachol and NMDA to induce action potentials in pyramidal neurons. The carbachol-induced firing frequency of layer V pyramidal cells was increased in *Ngfr*$^{-/-}$ mice as compared to control mice, whereas NMDA-induced firing frequency was not affected in *Ngfr*$^{-/-}$ mice. We argue that long-term reduction of cholinergic activity in *Ngfr*$^{-/-}$ mice may lead to some changes in the various cholinergic receptors in this region. This compensatory effect may lead to increased firing upon carbachol treatment in *Ngfr*$^{-/-}$ mice. However, in either carbachol-induced or NMDA-induced firing system, *Ngfr* deficiency resulted in consistent disinhibition to GABAergic agonist isoguvacine (**Fig. 6g and Supplementary Fig. 7c**). We have discussed these data in the Results section in the revised manuscript.

*9. Figure 7: saccharin solution and saccharin solution + nicotine should be administered also to Ngfr+/+ mice.*
**[Response]** We have examined the effect of chronic nicotine treatment in WT (*Ngfr*$^{+/+}$) mice and found no significant changes on temporal order recognition (new **Supplementary Fig.7g**). However, nicotine treatment rescued impaired temporal order recognition in *Ngfr*$^{-/-}$ mice (**Fig. 7f, g**). These data are consistent with the idea that deteriorated cholinergic activity accounts for the impaired recency judgements in *Ngfr*$^{-/-}$ mice.

**Reference**


1 Warburton, E. C. & Brown, M. W. Neural circuitry for rat recognition memory. *Behav Brain Res* **285**, 131-139, doi:10.1016/j.bbr.2014.09.050 (2015).
2 Boskovic, Z. *et al.* The role of p75NTR in cholinergic basal forebrain structure and function. *J Neurosci* **34**, 13033-13038, doi:10.1523/JNEUROSCI.2364-14.2014 (2014).
3 Boskovic, Z. *et al.* Cholinergic basal forebrain neurons regulate fear extinction consolidation through p75 neurotrophin receptor signaling. *Transl Psychiatry* **8**, 199, doi:10.1038/s41398-018-0248-x (2018).
4 Greferath, U. *et al.* Enlarged cholinergic forebrain neurons and improved spatial learning in p75 knockout mice. *Eur J Neurosci* **12**, 885-893, doi:10.1046/j.1460-9568.2000.00976.x (2000).

5 Meier, S. *et al.* The p75 neurotrophin receptor is required for the survival of neuronal progenitors and normal formation of the basal forebrain, striatum, thalamus and neocortex. *Development* **146**, doi:10.1242/dev.181933 (2019).

6 Naumann, T. *et al.* Complete deletion of the neurotrophin receptor p75NTR leads to long-lasting increases in the number of basal forebrain cholinergic neurons. *J Neurosci* **22**, 2409-2418, doi:10.1523/JNEUROSCI.22-07-02409.2002 (2002).

7 Peterson, D. A., Dickinson-Anson, H. A., Leppert, J. T., Lee, K. F. & Gage, F. H. Central neuronal loss and behavioral impairment in mice lacking neurotrophin receptor p75. *J Comp Neurol* **404**, 1-20, doi:10.1002/(sici)1096-9861(19990201)404:1<1::aid-cne1>3.0.co;2-# (1999).

8 Peterson, D. A., Leppert, J. T., Lee, K. F. & Gage, F. H. Basal forebrain neuronal loss in mice lacking neurotrophin receptor p75. *Science* **277**, 837-839, doi:10.1126/science.277.5327.837 (1997).

9 Ward, N. L. & Hagg, T. p75(NGFR) and cholinergic neurons in the developing forebrain: a re-examination. *Brain Res Dev Brain Res* **118**, 79-91, doi:10.1016/s0165-3806(99)00133-9 (1999).

10 Yeo, T. T. *et al.* Absence of p75NTR causes increased basal forebrain cholinergic neuron size, choline acetyltransferase activity, and target innervation. *J Neurosci* **17**, 7594-7605, doi:10.1523/JNEUROSCI.17-20-07594.1997 (1997).

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

All questions which I mentioned have been addressed by the authors.

Reviewer #2 (Remarks to the Author):

General comments:

The large parts of the manuscript were revised appropriately according to the reviewer's suggestions. In contrast, there are still some minor comments to the authors' responses that are not enough to satisfy the reviewer. The additional comments are described below each response.

1. The authors described that Ngfr-positive cholinergic neurons projecting from the SI/nBM to the mPFC selectively regulate temporal order recognition memory. This study indicated the importance of NGFR signaling in temporal order recognition memory by using the knockout mice, but SI/nBM neurons may be also involved in novel object recognition memory as reported in other studies (Okada et al., 2015). Therefore, it is unclear whether SI/nBM neurons projecting to the mPFC selectively contribute to the temporal order recognition. Chemogenetic or optogenetic inhibition experiments may be needed to test the novel object recognition.

[Response] We appreciated the reviewer’s comment and have now shown that the SI/nBM-mPFC cholinergic circuit selectively regulates temporal order but not novel object recognition memory. Specifically, we altered the SI/nBM-mPFC circuit using AAV-retro virus delivering hM4D(Gi) in ChAT-Cre mice. Bilateral injection of the AAV-retro virus into the mPFC enabled its retrograde transportation to SI/nBM, and specifically manipulated the ChAT-expressing and mPFC projecting SI/nBM cholinergic neurons (Fig. 5a and new Supplementary Fig. 5a). Chemogenetic inhibition of the SI/nBM-mPFC circuit significantly impaired temporal order recognition (Fig. 5b), but NOT the novel object recognition memory (new Supplementary Fig. 5b). This observation is consistent with previous studies showing that cortical lesions in the perirhinal cortex (PRH), but not mPFC, lead to novel object recognition memory deficits 1. Thus, while SI/nBM cholinergic neurons are involved in both forms of recognition memory, the SI/nBM-mPFC circuit controls temporal order whereas SI/nBM-PRH projection controls novel object recognition.

[Comment]

Throughout the whole manuscript, it is difficult to find the revised parts in the text. The authors should show the page and line numbers of changed parts in the revised manuscript.

Pathway-specific manipulation of cholinergic system indicated that the SI/nBM-mPFC route regulates temporal order recognition memory, but not novel object recognition memory.

In Supplementary Fig. 5, it is desirable to include the data in the sample phase.

2. The authors mentioned that Ngfr-positive cholinergic neurons regulate temporal order recognition memory through controlling GABAergic transmission. This study demonstrated the impaired depolarizing shift of GABAergic inputs to mPFC pyramidal neurons in the knockout mice by using electrophysiological recordings. However, the role of GABAergic regulation of pyramidal neurons in recognition memory has not been tested. Therefore, there is no direct evidence that GABAergic regulation of pyramidal neurons controls the recognition memory.

[Response] We appreciated the reviewer for this question, which helped us to improve the entire logic chain in the revised manuscript. We have shown that a reduction in KCC2 expression resulted in a depolarizing shift of GABAergic inputs to mPFC pyramid neurons in the Ngfr-/- mice, leading to a selective impairment in temporal order recognition (Fig. 6). Further, we bilaterally injected furosemide through cannula, a well-established KCC2 inhibitor, into the mPFC of adult wild-type (WT) mice at ages of 3 months and performed three types of recognition memory tests. Remarkably, mPFC delivery of furosemide specifically attenuated temporal order recognition (new Supplementary Fig. 7d), but NOT novel object recognition or object location memory (new Supplementary Fig. 7e, f). Our findings provide direct evidence that KCC2-mediated GABAergic driving force on mPFC pyramidal neurons controls recognition memory and strengthens the specific role of mPFC in the temporal order recognition, but not in the novel object and object location recognition.

[Comment]

The experimental conditions for intracranial injection of furosemide should be described in the methods section, such as coordinates of the cannula placement, injection volume and rate, and interval between the injection and behavioral experiment.

In Supplementary Fig. 7, it is desirable to include the data in the sample phase.

Since furosemide is a non-selective inhibitor of CCCs (Löscher et al., 2013), it remains uncertain whether the results of the pharmacological experiment are derived from selective inhibition of KCC2 in the mPFC. The authors should use a more selective inhibitor for KCC2 or the knockdown of

KCC2 by using shRNA expression because Ngfr knockout chronically affects gene expression level of KCC2.

If the results of the pharmacological experiment are hypothesized to be originating from selective inhibition of KCC2, the data would show that KCC2 function in mPFC pyramidal neurons is involved in temporal order recognition memory. In contrast, biochemical and electrophysiological studies show that NGFR signaling acts to maintain the expression level of KCC2 and affect GABAergic driving force, resulting in changes in firing activity of pyramidal neurons. These evidences suggest that KCC2-mediated regulation of GABAergic input in pyramidal neurons may be engaged to the control of recognition memory. The authors describe that "Our findings provide direct evidence that KCC2-mediated GABAergic driving force on mPFC pyramidal neurons controls recognition memory" (lines 466-468), but this reviewer does not consider that the direct evidence leading to this conclusion is presented herein. They should mention the conclusion more carefully.

3. ACh release was increased at the time window around object contacts in all phases (sample and test phases) of the temporal order recognition task in the control mice, whereas the release level was significantly decreased in all phases in the knockout mice (Figure 4). ACh release seems to be increased in association with object contacts. How do you explain the mechanism of recognition memory by increased ACh release? In other recognition memory tasks, the condition at the sample phase is the same as one in the temporal order recognition task. Thus, the release response would be similar between different recognition memory tasks. The data showing the normal behavior of knockouts on other recognition memory tasks suggest that Ach release at the sample phase is not important for recognition memory. The authors should measure ACh release at the test phase of other recognition memory tasks. The release response at the test phase may be different among the recognition memory tasks with or without the effects of the knockout.

[Response] The reviewer raised a series of questions regarding the physiological significance of increased ACh release in the mPFC and the underlying mechanism of recognition memory. The following efforts were made to address these questions.

(1) We have provided a video to show that ACh signal surges shortly prior to object encounter and rapidly declines when the mice withdrawal from exploration, suggesting that the ACh signal may function as an initiator for object recognition (new Supplementary video 1).

(2) We have quantitatively compared the strength of ACh release when the mice explore the earlier acquaintance (green circles) or the later acquaintance (purple squares) objects (new Fig. 4a). The ACh signals in the sample phase 1 and 2, in which the mice explored two identical objects, were equal (new Fig. 4e, left and middle). Remarkably, during the test phase, the ACh release was significantly higher when the mice explored the objects that they had encountered earlier, compared to those they had encountered later (new Fig. 4e, right). These data suggest that ACh signal not only predicts object encounter, but also directly determines recency judgments of objects.

(3) We systematically examined dynamic changes of ACh release in the mPFC during novel object and object location recognition. In contrast to temporal order recognition, ACh release in the mPFC of WT mice during novel object recognition was detected only in the sample phase (new Supplementary Fig.4a, b, c, d, left), but not in the test phase (new Supplementary Fig.4a, b, c, d, right). Likewise, in the object location recognition, elevated ACh signals were seen only in the sample phase (new Supplementary Fig.4e, f, g, h, left) but not in the test phase (new Supplementary Fig.4e, f, g, h, left). These data suggest that ACh signals in the mPFC are not involved in the memory retrieval of novel object and object location recognition.

(4) Although Ngfr-/- mice exhibited reduced ACh signals in the sample phase of novel object and object location recognition (new Supplementary Fig.4b-d, f-h), the behavioral outcomes of these two types of recognition remained intact. We speculate that defected ACh release during the encoding stage of recognition may not be sufficient to interrupt discrimination of object familiarity or spatial recognition. Indeed, lesions in the mPFC per se do not affect novel object and object location recognition 1.Taken together, our findings highlight the pivotal role of the mPFC and Ngfr-dependent ACh release in the mPFC in temporal order recognition memory, but not in novel object and object location recognition.

[Comment]

In the wild type mice, ACh release level in the mPFC is different between the two objects related to the recency in the test phase, whereas the difference in Ach release in the test phase is not observed in other recognition memory tasks. The data support that Ach release in the mPFC is associated with temporal order recognition memory.

In contrast, mPFC Ach release in the knockout mice is decreased in the sample phase in the novel object and object location recognition memory tasks but showing normal performance of these memory tasks. The authors suggest that the reduction in Ach release may not be sufficient for the impairments in the task performance. This suggestion may mean that a low level of Ach release is enough for the performance of the tasks. However, as described by the authors mPFC function is not required for novel object and object location recognition memory. For these memory tasks, they need to measure Ach release in other brain regions, such as PRH or hippocampus, which are reported to be necessary for the tasks. This issue is expected to be done in the next step in the future.

In Fig. 4, the procedure to calculate ACh release for each object in the test phase should be described in the Methods section.

As for “Ngfr-dependent ACh release” (lines 576-578), it remains unclear how NGFR signaling regulates Ach release in the mPFC. Discussion of the potential mechanism to explain Ngfr-dependent regulation of Ach secretion is useful for readers.

4. p.10, line 5: KCC2 level is largely different between the knockouts in Figure 6, panel h (~0.5) and the controls in Figure 7, panel a (~1.0).

[Response] In the Fig. 7a in the previous manuscript, we normalized the control treatment of Ngfr-/- mice as "1" and presented KCC2 levels of nicotine treatment group as fraction of the control. According to the review's suggestion, we normalized nicotine treatment as "1" and calculated the KCC2 levels of controls as fraction of the nicotine treatment (new Fig. 7a).

[Comment]

The procedure for normalization is necessary to be described in the Methods section.

Reviewer #3 (Remarks to the Author):

The study by Mei et al. is an important contribution to the field and is likely of interest to a broad readership of neuroscientists. The revised manuscript is much improved from the first submission and presents some exciting new data. Although the authors addressed all the reviewer concerns, there are still minor issues that should be addressed in the text before it is acceptable for publication. These are listed below.

1) In the discussion, line 594 please change "numbers of " with "inputs impinging on"

2) It seems that an important citation about nicotinic effect on KCC2 expression is missing: Liu et al., Science, volume 314, 2006.

3) Legend Supplementary figure 7 d-e-f, please specify wild-type mice

4) Supplementary Fig 6 All graphs with sIPSCs and sEPSCs should be labeled with sIPSP and sEPSC (frequency, amplitude). The plural is appropriate in the text but it should be singular in graph labels.

**REVIEWER COMMENTS**

**Reviewer #1 (Remarks to the Author):**

*All questions which I mentioned have been addressed by the authors.*
We appreciate the reviewer for this comment.

**Reviewer #2 (Remarks to the Author):**

*General comments:*
*The large parts of the manuscript were revised appropriately according to the reviewer's suggestions. In contrast, there are still some minor comments to the authors' responses that are not enough to satisfy the reviewer. The additional comments are described below each response.*
We thank the reviewer for the comments and suggestions. In the following, we are answering the questions point-by-point. The first-round and second-round reviewer comments are displayed in red italics, the first-round author response is presented in blue italics, and the second-round author response is shown in black.

*1. The authors described that Ngfr-positive cholinergic neurons projecting from the SI/nBM to the mPFC selectively regulate temporal order recognition memory. This study indicated the importance of NGFR signaling in temporal order recognition memory by using the knockout mice, but SI/nBM neurons may be also involved in novel object recognition memory as reported in other studies (Okada et al., 2015). Therefore, it is unclear whether SI/nBM neurons projecting to the mPFC selectively contribute to the temporal order recognition. Chemogenetic or optogenetic inhibition experiments may be needed to test the novel object recognition.*
*[Response] We appreciated the reviewer's comment and have now shown that the SI/nBM-mPFC cholinergic circuit selectively regulates temporal order but not novel object recognition memory. Specifically, we altered the SI/nBM-mPFC circuit using AAV-retro virus delivering hM4D(Gi) in ChAT-Cre mice. Bilateral injection of the AAV-retro virus into the mPFC enabled its retrograde transportation to SI/nBM, and specifically manipulated the ChAT-expressing and mPFC projecting SI/nBM cholinergic neurons (Fig. 5a and new Supplementary Fig. 5a). Chemogenetic inhibition of the SI/nBM-mPFC circuit significantly impaired temporal order recognition (Fig. 5b), but NOT the novel object recognition memory (new Supplementary Fig. 5b). This observation is consistent with previous studies showing that cortical lesions in the perirhinal cortex (PRH), but not mPFC, lead to novel object recognition memory deficits 1. Thus, while SI/nBM cholinergic neurons are involved in both forms of recognition memory, the SI/nBM-mPFC circuit controls temporal order whereas SI/nBM-PRH projection controls novel object recognition.*

*[Comment]*

*Throughout the whole manuscript, it is difficult to find the revised parts in the text. The authors should show the page and line numbers of changed parts in the revised manuscript.*

**[Response]** We apologize for this inconvenience and have shown the page and line numbers of changed parts in this second-round revision.

*Pathway-specific manipulation of cholinergic system indicated that the SI/nBM-mPFC route regulates temporal order recognition memory, but not novel object recognition memory.*

*In Supplementary Fig. 5, it is desirable to include the data in the sample phase.*

**[Response]** We thank the reviewer for this comment and have included the data in the sample phase (**new Supplementary Fig. 5b, 5d**). There is no significant difference in the recognition of two identical objects in the sample phase, in which the discrimination ratio is close to "0".

*2. The authors mentioned that Ngfr-positive cholinergic neurons regulate temporal order recognition memory through controlling GABAergic transmission. This study demonstrated the impaired depolarizing shift of GABAergic inputs to mPFC pyramidal neurons in the knockout mice by using electrophysiological recordings. However, the role of GABAergic regulation of pyramidal neurons in recognition memory has not been tested. Therefore, there is no direct evidence that GABAergic regulation of pyramidal neurons controls the recognition memory.*

*[Response] We appreciated the reviewer for this question, which helped us to improve the entire logic chain in the revised manuscript. We have shown that a reduction in KCC2 expression resulted in a depolarizing shift of GABAergic inputs to mPFC pyramid neurons in the Ngfr-/- mice, leading to a selective impairment in temporal order recognition (Fig. 6). Further, we bilaterally injected furosemide through cannula, a well-established KCC2 inhibitor, into the mPFC of adult wild-type (WT) mice at ages of 3 months and performed three types of recognition memory tests. Remarkably, mPFC delivery of furosemide specifically attenuated temporal order recognition (new Supplementary Fig. 7d), but NOT novel object recognition or object location memory (new Supplementary Fig. 7e, f). Our findings provide direct evidence that KCC2-mediated GABAergic driving force on mPFC pyramidal neurons controls recognition memory and strengthens the specific role of mPFC in the temporal order recognition, but not in the novel object and object location recognition.*

*[Comment]*

*The experimental conditions for intracranial injection of furosemide should be described in the methods section, such as coordinates of the cannula placement, injection volume and rate, and interval between the injection and behavioral experiment.*

**[Response]** We have described the experimental details for cannula implantation and the mPFC delivery of furosemide in page 20, lines 865-875 in the Methods section.

*In Supplementary Fig. 7, it is desirable to include the data in the sample phase.*

**[Response]** We have included the data in the sample phase (**new Supplementary Fig. 7d, e, f and 7g**). These data suggest no significant difference in the discrimination of two identical objects in the sample phase between different treatments.

*Since furosemide is a non-selective inhibitor of CCCs (Löscher et al., 2013), it remains uncertain whether the results of the pharmacological experiment are derived from selective inhibition of KCC2 in the mPFC. The authors should use a more selective inhibitor for KCC2 or the knockdown of KCC2 by using shRNA expression because Ngfr knockout chronically affects gene expression level of KCC2.*

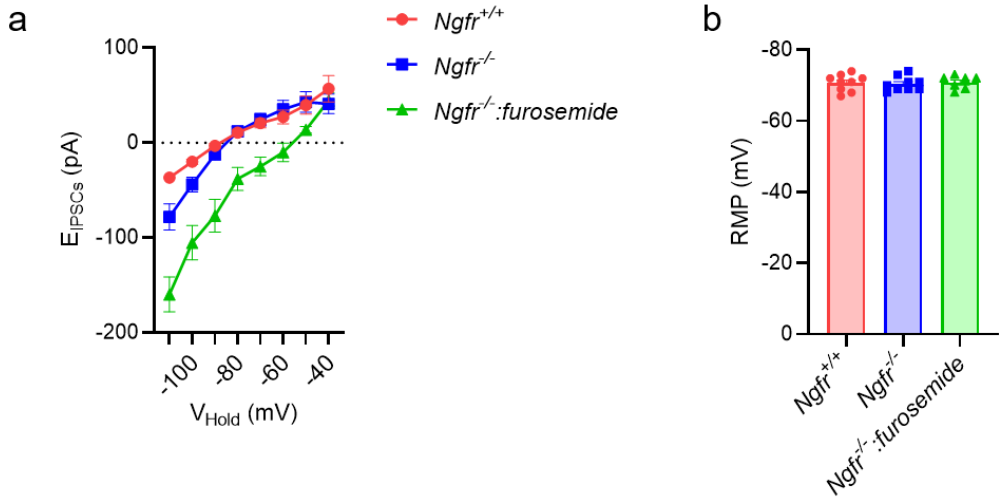
**[Response]** We appreciate the reviewer for this comment. The following efforts have been made to address the points related to the comments.

(1) Regarding to the original question that whether GABAergic regulation of pyramidal neurons controls recognition memory, previous studies have shown that a depolarizing shift of GABAergic signaling in the hippocampus, either by decreased KCC2 expression or increased NKCC1 expression, significantly impaired object location recognition memory [1,2]. These studies are consistent with our findings that the polarity of GABAergic inputs to pyramidal neurons is pivotal for recognition memory.

(2) In this study, we clearly demonstrated a depolarizing shift of GABAergic polarity and prominent GABAergic disinhibition in the mPFC of *Ngfr*$^{-/-}$ mice. To determine whether changes in the polarity of GABAergic signaling in the *Ngfr*$^{-/-}$ mPFC contribute to the impairment in temporal order recognition, we used the inhibitor of chloride extruder KCC2, furosemide, to alter the GABAergic driving force in the WT mPFC. As the reviewer pointed out, the selectivity of furosemide may not be high enough, and it may also inhibit the chloride intruder NKCC1. However, we found that delivery of furosemide to the mPFC of adult WT mice specifically attenuated temporal order recognition, but not novel object recognition or object location recognition (Supplementary Fig. 7d, e, f), an effect similar to that seen in *Ngfr*$^{-/-}$ mice with reduced KCC2 expression in the mPFC (Fig. 3a-k and 6h). Logically, if furosemide also inhibits NKCC1, it should elicit the opposite effect, which is not what we saw. Finally, we found a reduction in KCC2 expression, but intact NKCC1 levels, was associated with the depolarizing shift in GABAergic polarity and impairment in temporal order recognition (Fig. 6h, i). Taken together, we believe that mPFC delivery of furosemide, which may affect other molecules in certain circumstances, attenuated temporal order recognition by inhibiting KCC2.

(3) We have provided additional data showing that furosemide treatment in the brain slices indeed resulted in a depolarizing shift of $E_{IPSCs}$, resembling the effect of downregulated KCC2 but not NKCC1. In the *Ngfr*$^{-/-}$ hippocampus, where the cholinergic activity of the MS-hippocampus pathway was intact (Fig. 2d, e), the reversal potential of $E_{IPSCs}$ was the same between the two genotypes (Fig. 6j, k, l). However, bath application of 50 μM furosemide resulted in a dramatic depolarizing shift of $E_{IPSCs}$ (**Response Figure 1**, **below**), suggesting that furosemide alters GABAergic driving force through inhibition of the chloride extruder KCC2.

Thus, the fact that furosemide may elicit potential side effect on other molecules, such as NKCC1, does not undermine the core assertion that a depolarizing shift in GABAergic signaling via KCC2 inhibition/reduction in the mPFC impairs temporal order recognition. In light of the potential non-selective effect of furosemide, we have reinterpreted the results (page 11, lines 444-466) and refined the discussion (page 15, lines 623-630) in the revised manuscript.




**Response Figure 1**. Depolarizing shift of the reversal potential of $E_{IPSCs}$ by furosemide treatment. **a** There was no significant difference in the reversal potential of $E_{IPSCs}$ in the hippocampal CA1 neurons between *Ngfr*$^{+/+}$ and *Ngfr*$^{-/-}$ mice. Bath application of 50 μM furosemide induces a notable depolarizing shift of $E_{IPSCs}$ in *Ngfr*$^{-/-}$ mice. **b** No significant difference in resting membrane potential (RMP) between groups. *Ngfr*$^{+/+}$, n=9 cells from 3 mice; *Ngfr*$^{-/-}$, n=9 cells from 3 mice; *Ngfr*$^{-/-}$ with furosemide, n=7 cells from 2 mice.

*If the results of the pharmacological experiment are hypothesized to be originating from selective inhibition of KCC2, the data would show that KCC2 function in mPFC pyramidal neurons is involved in temporal order recognition memory. In contrast, biochemical and electrophysiological studies show that NGFR signaling acts to maintain the expression level of KCC2 and affect GABAergic driving force, resulting in changes in firing activity of pyramidal neurons. These evidences suggest that KCC2-mediated regulation of GABAergic input in pyramidal neurons may be engaged to the control of recognition memory. The authors describe that "Our findings provide direct evidence that KCC2-mediated GABAergic driving force on mPFC pyramidal neurons controls recognition memory" (lines 466-468), but this reviewer does not consider that the direct evidence leading to this conclusion is presented herein. They should mention the conclusion more carefully.*

**[Response]** We appreciate the reviewer for this comment. In the revised manuscript, we have adjusted the logic flow regarding the mechanism underlying the impaired temporal order recognition memory in *Ngfr*$^{-/-}$ mice. First, we demonstrate a prominent

depolarizing shift of GABAergic driving force in the *Ngfr*$^{-/-}$ mPFC. Second, we examined the impact of the altered mPFC GABAergic signaling on recognition memory. By furosemide treatment to interfere with GABAergic driving force in the WT mice, we showed that temporal order recognition memory was selectively impaired. These data indicate that disturbance in the polarity of GABAergic signaling in the mPFC deteriorates temporal order recognition. Third, we delineated that downregulated KCC2 expression, but intact NKCC1 levels, was associated with the depolarizing shift of GABAergic signaling in the *Ngfr*$^{-/-}$ mice.

Based on these experiments, we hypothesized that the polarity of GABAergic inputs on the mPFC pyramidal neurons regulates temporal order recognition memory. Specifically, reduced KCC2 levels in the *Ngfr*$^{-/-}$ mice is associated with depolarizing shift of the GABAergic signaling. We have tuned down our interpretation and deleted the sentence "Our findings provide direct evidence that KCC2-mediated GABAergic driving force on mPFC pyramidal neurons controls recognition memory." in the revised manuscript.

*3. ACh release was increased at the time window around object contacts in all phases (sample and test phases) of the temporal order recognition task in the control mice, whereas the release level was significantly decreased in all phases in the knockout mice (Figure 4). ACh release seems to be increased in association with object contacts. How do you explain the mechanism of recognition memory by increased ACh release? In other recognition memory tasks, the condition at the sample phase is the same as one in the temporal order recognition task. Thus, the release response would be similar between different recognition memory tasks. The data showing the normal behavior of knockouts on other recognition memory tasks suggest that Ach release at the sample phase is not important for recognition memory. The authors should measure ACh release at the test phase of other recognition memory tasks. The release response at the test phase may be different among the recognition memory tasks with or without the effects of the knockout.*

*[Response] The reviewer raised a series of questions regarding the physiological significance of increased ACh release in the mPFC and the underlying mechanism of recognition memory. The following efforts were made to address these questions.*

*(1) We have provided a video to show that ACh signal surges shortly prior to object encounter and rapidly declines when the mice withdrawal from exploration, suggesting that the ACh signal may function as an initiator for object recognition (new Supplementary video 1).*

*(2) We have quantitatively compared the strength of ACh release when the mice explore the earlier acquaintance (green circles) or the later acquaintance (purple squares) objects (new Fig. 4a). The ACh signals in the sample phase 1 and 2, in which the mice explored two identical objects, were equal (new Fig. 4e, left and middle). Remarkably, during the test phase, the ACh release was significantly higher when the mice explored the objects that they had encountered earlier, compared to those they had encountered later (new Fig. 4e, right). These data suggest that ACh signal not only predicts object encounter, but also directly determines recency judgments of objects.*

*(3) We systematically examined dynamic changes of ACh release in the mPFC during novel object and object location recognition. In contrast to temporal order recognition, ACh release in the mPFC of WT mice during novel object recognition was detected only in the sample phase (new Supplementary Fig.4a, b, c, d, left), but not in the test phase (new Supplementary Fig.4a, b, c, d, right). Likewise, in the object location recognition, elevated ACh signals were seen only in the sample phase (new Supplementary Fig.4e, f, g, h, left), but not in the test phase (new Supplementary Fig.4e, f, g, h, left). These data suggest that ACh signals in the mPFC are not involved in the memory retrieval of novel object and object location recognition.*
*(4) Although Ngfr-/- mice exhibited reduced ACh signals in the sample phase of novel object and object location recognition (new Supplementary Fig.4b-d, f-h), the behavioral outcomes of these two types of recognition remained intact. We speculate that defected ACh release during the encoding stage of recognition may not be sufficient to interrupt discrimination of object familiarity or spatial recognition. Indeed, lesions in the mPFC per se do not affect novel object and object location recognition 1.Taken together, our findings highlight the pivotal role of the mPFC and Ngfr-dependent ACh release in the mPFC in temporal order recognition memory, but not in novel object and object location recognition.*

*[Comment]*
*In the wild type mice, ACh release level in the mPFC is different between the two objects related to the recency in the test phase, whereas the difference in Ach release in the test phase is not observed in other recognition memory tasks. The data support that Ach release in the mPFC is associated with temporal order recognition memory.*

*In contrast, mPFC Ach release in the knockout mice is decreased in the sample phase in the novel object and object location recognition memory tasks but showing normal performance of these memory tasks. The authors suggest that the reduction in Ach release may not be sufficient for the impairments in the task performance. This suggestion may mean that a low level of Ach release is enough for the performance of the tasks. However, as described by the authors mPFC function is not required for novel object and object location recognition memory. For these memory tasks, they need to measure Ach release in other brain regions, such as PRH or hippocampus, which are reported to be necessary for the tasks. This issue is expected to be done in the next step in the future.*
**[Response]** We thank the reviewer for the suggestion for future studies. We have added a description on the measurement of ACh release in different brain regions during novel object recognition and object location recognition in the Discussion section (pages 14, lines 587-589).

*In Fig. 4, the procedure to calculate ACh release for each object in the test phase should be described in the Methods section.*
**[Response]** We have described the procedure to calculate ACh release for each object in the sample phase and test phase in the Methods section (page 20, lines 840-842).

*As for "Ngfr-dependent ACh release" (lines 576-578), it remains unclear how NGFR signaling regulates Ach release in the mPFC. Discussion of the potential mechanism to explain Ngfr-dependent regulation of Ach secretion is useful for readers.*

**[Response]** We thank the reviewer for this comment, and have included a paragraph to discuss the potential mechanism underlying NGFR regulation of the excitability of cholinergic neurons and ACh secretion in the Discussion section (pages 13, lines 545-552).

*4. p.10, line 5: KCC2 level is largely different between the knockouts in Figure 6, panel h (~0.5) and the controls in Figure 7, panel a (~1.0).*

*[Response] In the Fig. 7a in the previous manuscript, we normalized the control treatment of Ngfr-/- mice as "1" and presented KCC2 levels of nicotine treatment group as fraction of the control. According to the review's suggestion, we normalized nicotine treatment as "1" and calculated the KCC2 levels of controls as fraction of the nicotine treatment (new Fig. 7a).*

*[Comment]*

*The procedure for normalization is necessary to be described in the Methods section.*

**[Response]** Thanks for the comment. We have described the procedure for normalization in the Methods section (pages 20, lines 828-830).

**Reference**


1 Deidda, G. *et al.* Reversing excitatory GABAAR signaling restores synaptic plasticity and memory in a mouse model of Down syndrome. *Nat Med* **21**, 318-326, doi:10.1038/nm.3827 (2015).

2 Simonnet, C. *et al.* Silencing KCC2 in mouse dorsal hippocampus compromises spatial and contextual memory. *Neuropsychopharmacology* **48**, 1067-1077, doi:10.1038/s41386-022-01480-5 (2023).

**Reviewer #3 (Remarks to the Author):**

*The study by Mei et al. is an important contribution to the field and is likely of interest to a broad readership of neuroscientists. The revised manuscript is much improved from the first submission and presents some exciting new data. Although the authors addressed all the reviewer concerns, there are still minor issues that should be addressed in the text before it is acceptable for publication. These are listed below.*
We appreciated the reviewer for this constructive comment.

*1) In the discussion, line 594 please change "numbers of" with "inputs impinging on"*
**[Response]** We thank the reviewer for pointing out this error. We have corrected it (page 15, line 607).

*2) It seems that an important citation about nicotinic effect on KCC2 expression is missing: Liu et al., Science, volume 314, 2006.*
**[Response]** This citation is listed as 42$^{th}$ in the Reference and cited in page 11, line 450 and page 15, line 617.

*3) Legend Supplementary figure 7 d-e-f, please specify wild-type mice*
**[Response]** We have specified the furosemide treatment in the wild-type mice in page 35, lines 1496-1500 of Supplementary Fig. 7 d. e. f legend.

*4) Supplementary Fig 6 All graphs with sIPSCs and sEPSCs should be labeled with sIPSP and sEPSC (frequency, amplitude). The plural is appropriate in the text but it should be singular in graph labels.*
**[Response]** We thank the reviewer for this comment and have changed the plural to singular in graph labels in Supplementary Fig. 6.