Research report

Inhibition of Piezo1/Ca\(^{2+}\)/calpain signaling in the rat basal forebrain reverses sleep deprivation-induced fear memory impairments

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ABSTRACT

In this study, we tested the hypothesis that the Piezo1/Ca\(^{2+}\)/calpain pathway of the basal forebrain (BF) modulates impaired fear conditioning caused by sleep deprivation. Adult male Wistar rats were subjected to 6 h of total sleep deprivation using the gentle handling protocol. Step-down inhibitory avoidance tests revealed that sleep deprivation induced substantial short- and long-term fear memory impairment in rats, which was accompanied by increased Piezo1 protein expression (\(P < 0.01\)) and increased cleavage of full-length tropomyocin receptor kinase B (TrkB-FL) (\(P < 0.01\)) in the BF area. Microinjection of the Piezo1 activator Yoda1 into the BF mimicked these sleep deprivation-induced phenomena; TrkB-FL cleavage was increased (\(P < 0.01\)) and short- and long-term fear memory was impaired (both \(P < 0.01\)) by Yoda1. Inhibition of Piezo1 by GsMTx4 in the BF area reduced TrkB-FL degradation (\(P < 0.01\)) and partially reversed short- and long-term fear memory impairments in sleep-deprived rats (both \(P < 0.01\)). Inhibition of calpain activation, downstream of Piezo1 signaling, also improved short- and long-term fear memory impairments (\(P = 0.038, P = 0.011\)) and reduced TrkB degradation (\(P < 0.01\)) in sleep-deprived rats. Moreover, sleep deprivation induced a lower pain threshold than the rest control, which was partly reversed by microinjection of GsMTx4 or PD151746. Neither sleep deprivation nor the abovementioned drugs affected locomotion and sedation. Taken together, these results indicate that BF Piezo1/Ca\(^{2+}\)/calpain signaling plays a role in sleep deprivation-induced TrkB signaling disruption and fear memory impairments in rats.

1. Introduction

Adequate sleep is imperative for optimized memory processes [1]. Moreover, patients that exhibit more preoperative sleep disturbances tend to develop delirium postoperatively [2]. Sleep deprivation also induces cognitive impairments in humans and animals [3,4]. The mammalian basal forebrain (BF) is home to many ascending and descending pathways involved in multiple brain functions, including sleep-wake regulation, control of attention, and memory processes [5–7]. However, the role of the BF in sleep deprivation-induced memory impairments remains unclear.

The full-length tropomyocin receptor kinase B (TrkB-FL) is a receptor for the brain-derived neurotrophic factor (BDNF) [8], and plays a key role in the pathophysiology of cognitive impairments and the therapeutic mechanisms of cognitive resuscitation strategies [9,10]. In our previous research, we partly reversed sleep deprivation-induced fear memory deficits by activating the BF BDNF/TrkB pathway [11]. Calpains are Ca\(^{2+}\)-dependent proteases, whose overactivation degrades TrkB-FL [12]. For example, the Amyloid-\(\beta\) peptide promotes an increase in intracellular calcium concentrations and the subsequent over-activation of calpains, which then promote TrkB-FL cleavage [13,14]. The process of TrkB-FL cleavage generates a membrane-bound truncated...
receptor (TrkB-T) and an intracellular fragment (TrkB-ICD) [15]. TrkB-FL cleavage and the presence of TrkB-ICD in human brain samples might contribute to the loss of BDNF signaling in several neurodegenerative diseases, such as Alzheimer’s disease in humans [15,16]. Therefore, part of this study involves characterizing the stability of TrkB-ICD to observe whether sleep deprivation leads to increased cleavage of TrkB-FL [15].

Piezo1 is a newly discovered mechanically sensitive cation channel. In our previous studies, we regulated Piezo1 with the inhibitor GsMTx4 and successfully inhibited calpain-mediated neuronal apoptosis [17]. However, the ability of Piezo1 to regulate calpain-mediated TrkB-FL cleavage in the BF, and therefore modulate sleep deprivation-induced fear memory impairments, has not yet been clearly determined. Therefore, in this study, we perform sleep deprivation and step-down inhibitory avoidance experiments on adult male Wistar rats. In the experiments, we observe increased expression of Piezo1 and increased cleavage of TrkB-FL in the BF after acute sleep deprivation. Thus, we propose and test the hypothesis that the BF Piezo1/Ca^{2+}/calpain pathway modulates the impairments of fear conditioning caused by sleep deprivation.

2. Materials and methods

2.1. Animals and sleep deprivation

Adult male Wistar rats (300 ± 20 g) from Experimental Charles River Laboratories, Beijing, China, were used for this study. Procedures involving animals were conducted in accordance with the Guide for Care and Use of Laboratory Animals. All animals were housed individually under controlled conditions (12 h/12 h light-dark cycle, lights on at

![Experimental design](image1.png)

**Fig. 1.** Acute sleep deprivation induces Piezo1 protein expression and TrkB-FL cleavage in the rat basal forebrain. (A) Schematic design. Adult male Wistar rats were subjected to either 6 h of full sleep deprivation or rest control followed by inhibitory avoidance training. The effects of sleep deprivation on basal forebrain Piezo1 protein expression and TrkB-FL cleavage were evaluated 1 h after behavioral training. (B-D) Representative Western blot images and histograms indicating increased Piezo1 protein expression (B) and TrkB-FL cleavage (C) after sleep deprivation, but no change in TrkB-FL expression (C). (E and F) Representative immunohistochemical pictures (E) and histograms (F) showing increased Piezo1 expression in basal forebrain neurons after sleep deprivation. ***, P < 0.01. scale Bar = 50 μm. Data are expressed as means ± standard deviations.
08:00) in an isolated ventilated chamber maintained at 23 ± 1 °C, and provided access to food and water ad libitum. The experiments began when all animals had acclimated to the environmental conditions for one week.

Total sleep deprivation for 6 h was induced using the “gentle handling protocol”, which allows a larger range of natural behaviors than forced motion [18]. The rats were not disturbed but were handled with a soft writing brush when they closed their eyes or stopped whisking. Whisking is defined as movements of the whiskers that are typically synchronous with the eye movements. Perturbation strategies included using a soft writing brush to stir the bedding, a finger snap to make noise, and slightly tapping or rotating the cage. Sleep deprivation was initiated with onset of the light cycle (diurnal lighting conditions; 08:00–14:00) [19].

2.2. Experimental design

This study aimed to verify the hypothesis that the BF Piezo1/Ca²⁺/calpain pathway modulates the impairments of fear conditioning caused by sleep deprivation. To achieve this goal, this study comprised the following four parts. Firstly, the impact of sleep deprivation on BF Piezo1/Ca²⁺/calpain signaling activities was examined, including BF Piezo1 protein expression and TrkB cleavage (Fig. 1A). Animals were divided into a sleep deprivation group and a rest control group during this stage of the experiment.

Secondly, either the selective Piezo1 activator Yoda1 or the selective Piezo1 inhibitor GsMTx4 was microinjected bilaterally into the BF to observe the impacts of modulating Piezo1 signaling on sleep deprivation-induced TrkB-FL cleavage and memory impairments (Fig. 2A). GsMTx4 (ab141871) was purchased from Abcam, USA, and Yoda1 (SMI1558) was purchased from Sigma, Switzerland. Rats were randomly divided into six groups (n = 16 per group) and subjected to sleep deprivation or rest control combined with combined with GsMTx4 treatment, Yoda1 treatment, or no treatment. Specifically, 1 h after inhibitory avoidance training, 24 randomly selected rats (four rats per group) were euthanized to collect BF samples for Western blot examination of TrkB-FL cleavage and TrkB/PKC/PLCγ1 signaling activity. Seventy-two rats (12 rats per group) were subjected to both short-term memory and long-term memory tests. The rats were then sacrificed. Hematoxylin and eosin staining (HE) of the brain was performed to verify the injection site.

Thirdly, the activity of calpain, a downstream effector of Piezo1 signaling and the mediator of TrkB-FL cleavage, was inhibited by PD151746 to determine whether the rescue effects of Piezo1 inhibition on sleep deprivation-induced TrkB-FL cleavage and memory impairments rely on decreased calpain activity (Fig. 3A). PD151746 (ab145523) was purchased from Abcam, USA. Eighty rats were randomly divided into five groups (n = 16 per group) and subjected to sleep deprivation or rest control combined with either PD151746, PD151746 plus GsMTx4, or no treatment. One h after fear training, 20 randomly selected rats (four rats per group) were euthanized to collect BF samples for the Western blot examination of TrkB-FL cleavage and TrkB/PKC/PLCγ1 signaling activity. Sixty randomly selected rats (12 rats per group) were subjected to both short and long-term memory evaluation. Hematoxylin-eosin staining of the rat brains was performed to verify the injection sites.

Finally, to investigate whether sleep deprivation or drug injection altered other memory-related behaviors such as locomotor activity, pain perception and sedation, the open-field test, sedative evaluation, and manual von Frey test were conducted in sequence on 54 rats (Fig. 4A). The rats were divided into nine groups with six rats per group. The injection sites were checked visually after completion of the experiments.

2.3. Basal forebrain microinjection

Bilateral cannula implantation surgery was performed, where the guide tube tip was located 1 mm above the BF (relative to the Bregma: anterior-posterior (AP) – 1.06 mm, medial-lateral (ML) ± 2.57 mm, and dorsal-ventral (DV) – 9 mm) under general anesthesia, as described previously [11]. After 10 days of recovery, animals were either subjected to sleep deprivation or not, followed by inhibitory avoidance training. Either the Piezo1 inhibitor (GsMTx4) (250 μmol/L, 1 μL/side), Piezo1 activator (Yoda1) (300 μmol/L, 1 μL/side), calpain inhibitor PD151746 (2 mmol/L, 1 μL/side), PD151746 + GsMTx4 (1:1, 1 μL/side), or artificial cerebral spinal fluid (aCSF) (1 μL/side) was administrated 60 min prior to fear memory training. The aCSF served as the vehicle control, which contained (in mM) 125 NaCl, 3.5 KCl, 26 NaHCO₃, 1 MgCl₂, and 10 glucose (pH = 7.4, 315 mOsm/L) [20]. The selected doses were based on previous studies that reported the neuroprotective effects or neuronal injury effects of these drugs [17,21].

Bilateral microinjection into the BF was performed using a 10-μL Hamilton syringe (Hamilton, Switzerland) pumped by a microinfrusing pump (Harvard Apparatus, USA). The drug microinjection process lasted 2 min. After the injection, the syringe was left in place for 5 min to allow diffusion. The correct microinjection sites were confirmed by visual inspection or HE staining. Only data from rats with correct injection sites were used (Fig. 5).

2.4. Step-down inhibitory avoidance test

The test was performed in a plastic cubic box (50×50×25 cm) containing one rubber safe platform fixed to the floor and 16 parallel copper bars connected to an electric stimulator. Briefly, the animals were habituated to the apparatus for 30 s, allowing free exploration. Then, the animals were subjected to a training session where they were placed on the safe platform. Immediately after the animal stepped down with four legs on the grids, an electric shock of 0.4 mA was applied for 2 s. Each animal was placed again on the safe platform 1 h after the training session. Latency to step down was recorded as the level of short-term memory retention. Long-term memory was evaluated 24 h after training. The maximum observation time was 300 s.

2.5. Locomotor activity evaluation

The open-field test was used to evaluate locomotor behaviors. Rats were placed in a 100 × 100 × 40 cm (W × D × H) arena with opaque sidewalls under dim lighting, the floor was divided into 25 squares measuring 20 cm each. The experimental sessions were recorded by a video camera. Each rat was placed in the central area and allowed to freely explore for 5 min; every time both hind paws entered one square, a crossing was recorded. After each test, the open-field was cleaned with a solution of 20% ethanol then dried with a cloth. The total distance ran by the animals was estimated from the number of squares crossed. The time spent in the central and peripheral areas (all squares next to the walls) was also recorded. Rearing was then analyzed.

2.6. Sedation evaluation

The sedative/anesthetic levels were evaluated using the methods described by Laalou and Gamou [22,23]. The scores were as follows: wakefulness (score 0) = spontaneous locomotor activity in 1 min of observation; light sedation (0.2) = no spontaneous locomotion in 1 min of observation; deep sedation (0.4) = no motor response when placed on a grid inclined at 45° with the head down during a 30-s period of observation; light anesthesia (0.6) = no righting reflex during a 30-s period of observation; moderate anesthesia (0.8) = no paw withdrawal reflex; deep anesthesia (1.0) = no eye-blink reflex. Rats were scored by blinded experimenters.

2.7. Von Frey test

Rats were placed in a plastic cage with a wire mesh bottom that
A Experimental design

- Canula implantation (n=96)
- 1-week recovery
- 6 h sleep deprivation or rest control
- Western blot for PIEZO1, and TrkB-FL/PLCγ1 signaling
- 1 h after training
- Fear memory test
- Inhibitory avoidance training
- Yoda1 (PIEZ01 activator) or vehicle or
  GsMTx4 (PIEZ01 inhibitor)
  Basal forebrain microinjection
  (1 h prior to the end of sleep deprivation)

B

- PIEZO1
- GAPDH
- Relative PIEZO1 expression
  Sleep + - - - -
  GSMTx4 - - - - -
  Yoda1 - - - - +

C

- p-TrkB
- TrkB
- Relative p-TrkB expression
  Sleep + - - - -
  GSMTx4 - - - - -
  Yoda1 - - - - +

D

- TrkB-ICD
- GAPDH
- Relative TrkB-ICD expression
  Sleep + - - - -
  GSMTx4 - - - - -
  Yoda1 - - - - +

E

- p-PLCγ1
- PLCγ1
- Relative p-PLCγ1 expression
  Sleep + - - - -
  GSMTx4 - - - - -
  Yoda1 - - - - +

F

- Short-term memory
- Step-down latency time (sec)
- Sleep + - - - -
  GSMTx4 - - - - -
  Yoda1 - - - - +

G

- Long-term memory
- Step-down latency time (sec)
- Sleep + - - - -
  GSMTx4 - - - - -
  Yoda1 - - - - +

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allowed full access to the paws. Behavioral accommodation was allowed for approximately 15 min, until cage exploration and major grooming activities ceased. The area tested was the mid-plantar left hind paw, in the sciatic nerve distribution, avoiding the less sensitive footpads. The paw was touched with one of a series of eight von Frey hairs with logarithmically incremental stiffness. The von Frey hair was presented perpendicular to the plantar surface with sufficient force to cause slight buckling against the paw, and held for approximately 6–8 s. Stimuli were presented at intervals of several seconds, allowing for apparent resolution of any behavioral responses to previous stimuli. A positive response was noted if the paw was sharply withdrawn. Flinching immediately upon removal of the hair was also considered a positive response. Ambulation was considered an ambiguous response, and in such cases the stimulus was repeated. Stimuli were always presented in a consecutive fashion, whether ascending or descending. In the absence of a paw withdrawal response to the initially selected hair, a stronger stimulus was presented. In the event of paw withdrawal, the next weaker stimulus was chosen. Optimal threshold calculation by this method requires six responses. The resulting pattern of positive and negative responses was tabulated using the following convention: X = withdrawal; 0 = no withdrawal. The up-down method of characterizing the response threshold in rats was examined using the six responses [24].

2.8. Tissue extraction

We extracted fresh BF tissue as described in a previous study [25]. Briefly, we obtained a 1-mm-thick coronal brain slice by cutting the anterior and posterior ends of the optic chiasm. Then, we generated a horizontal cut through the middle of the coronal slice between the anterior commissure and the bottom of the slice. Finally, we made a vertical cut 1 mm lateral to the third ventricle and the middle of the olfactory tubercle on both sides. The obtained BF tissue (1 × 1 × 2 mm) from each side contains the magnocellular preoptic area, the nucleus of the horizontal limb of the diagonal band, the substantia innominata, and the basal nucleus [25]. Tissues were stored at −80 °C until further tests.

2.9. Immunohistochemistry

BF tissue was extracted and fixed with 4% paraformaldehyde in PBS to investigate Piezo1 expression changes in the BF after 6 h of sleep deprivation. Then, coronal Section (5 μm) were cut in the optic chiasm and blocked with Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% goat serum for 1 h at room temperature. The slides were incubated overnight at 4 °C with a rabbit anti-Piezo1 antibody (1:1000) (ab128245, Abcam), rabbit anti-phosphorylated TrkB (p-TrkB) (1:1000) (ABN1381, Millipore), rabbit anti-TRX-R (1:2000) (4603, CST), rabbit anti-TRK-B (1:2000) (ab18987, Abcam), rabbit anti-phosphorylated PLCγ1 (p-PLCγ1) (1:1000) (ab76155, Abcam), rabbit anti-PLC (1:2000) (ab90718, Abcam), or GAPDH (1:20,000) (ab181603, Abcam). On the following day, membranes were washed then incubated for 1 h at room temperature with Tris-buffered saline containing 0.1% Tween 20 (TBST) and the horseradish peroxidase (HRP)-conjugated secondary antibody IgG-HRP (ab6721, Abcam). Finally, the blots were visualized with an electrochemiluminescence kit (GE, USA) and photographed using a Bio-Rad imaging system. The optical density of the bands was determined using Quantity One software (Biorad). The protein densities of Piezo1, TrkB-FL, and TrkB-ICD were normalized to GAPDH, the protein densities of p-TrkB and p-PLCγ1 were normalized to TrkB and PLCγ1, respectively, and the relative protein expression levels were compared among the groups.

2.10. Western blot

Proteins were extracted from the BF with a protein extraction kit (C5000007, Sangon, Biotech, China). Protein concentrations were then determined using a BCA protein quantification kit (C503051, Sangon Biotech, China). Equivalent amounts of proteins were loaded into each lane, separated on 12% SDS-PAGE gels, then transferred onto polyvinyl difluoride membranes (10600023, GE, US). After blocking, the membranes were incubated overnight at 4 °C with either a rabbit anti-Piezo1 antibody (1:1000) (ab128245, Abcam), rabbit anti-phosphorylated TrkB (p-TrkB) (1:1000) (ABN1381, Millipore), rabbit anti-TRX-R (1:2000) (4603, CST), rabbit anti-TRK-B (1:2000) (ab18987, Abcam), rabbit anti-phosphorylated PLCγ1 (p-PLCγ1) (1:1000) (ab76155, Abcam), rabbit anti-PLC (1:2000) (ab90718, Abcam), or GAPDH (1:20,000) (ab181603, Abcam). On the following day, membranes were washed then incubated for 1 h at room temperature with Tris-buffered saline containing 0.1% Tween 20 (TBST) and the horseradish peroxidase (HRP)-conjugated secondary antibody IgG-HRP (ab6721, Abcam). Finally, the blots were visualized with an electrochemiluminescence kit (GE, USA) and photographed using a Bio-Rad imaging system. The optical density of the bands was determined using Quantity One software (Biorad). The protein densities of Piezo1, TrkB-FL, and TrkB-ICD were normalized to GAPDH, the protein densities of p-TrkB and p-PLCγ1 were normalized to TrkB and PLCγ1, respectively, and the relative protein expression levels were compared among the groups.

2.11. Statistical analyses

Statistical analyses were performed using SPSS 18.0 software (IBM). As the behavioral test data did not follow a normal distribution, we ranked the data and analyzed them using two-way repeated measures analysis of variance (ANOVA) with the post hoc Tukey multiple comparisons test. Data are presented as the medians and interquartile ranges in scatter plot diagrams. Western blot and immunohistochemistry data are presented as the means ± standard deviations (SD). One-way ANOVA with post hoc Bonferroni’s test was used for pairwise comparisons. Statistical significance was set to P < 0.05.

3. Results

3.1. Localization of microinjection sites in the basal forebrain

Fig. 5 summarizes the microinjection sites of the rats used in this study. The microinjection sites were confirmed by either HE staining after the behavioral assessment or visual inspection when the brains were sampled for Western blot analysis. Initially, 221 rats were used. Inspection of the microinjection sites found 176 rats with correct microinjection sites (BF), whose data were used in subsequent analysis.
Fig. 3. Inhibition of calpain activation in the basal forebrain mitigates Trk-FL cleavage, increases PLCγ1 phosphorylation, and partly recovers short- and long-term fear memory impairments induced by sleep deprivation. (A) Schematic design. Adult male Wistar rats were subjected to 6 h of total sleep deprivation or rest control followed by inhibitory avoidance training. The selective calpain inhibitor PD151746 (2 mmol/L, 1 μL/side), Piezo1 inhibitor GsMTx4 (250 μmol/L, 1 μL/side) or vehicle control were microinjected bilaterally into the basal forebrain 1 h prior to the end of sleep deprivation. The effects of basal forebrain calpain inhibition on TrkB-PLCγ1 signaling activity and short-and long-term impairments were then detected. (B-E) Representative Western blot images (B) and histograms showing the partial rescue of changes in TrkB phosphorylation (p-TrkB), TrkB-FL cleavage (TrkB-ICD), and PLCγ1 phosphorylation (p-PLCγ1) after sleep deprivation by PD151746 or GsMTx4. PD151746 plus GsMTx4 exhibited no synergistic effects. (F-G) Box and whiskers plots (min to max) combined with scatter plots showing the partial rescue of sleep deprivation induced short- (F) and long- (G) term fear memory impairments by GsMTx4. * P < 0.05; ** P < 0.01 compared to the rest control group. # P < 0.05; ## P < 0.01 compared to the sleep deprivation group. & & P < 0.01 compared to the PD151746 group. Data were expressed as means ± standard deviations for the Western blot results. n = 4 per group for the Western blot experiment. N = 12 per group for the memory test.
A Experimental design

B

C

D

E

F

1 week recovery

6 h sleep deprivation or rest control

Yoda1 (PIEZO1 activator) or
GSMTx4 (PIEZO1 inhibitor) or
PD151746 (calpain inhibitor) or
vehicle or
PD151746+GSMTx4

basal forebrain microinjection
(1 h prior to the end of sleep deprivation)

1 h after sleep deprivation

Locomotor activity evaluation Von Frey pain test

Sedation evaluation

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We then sacrificed 61 rats to further investigate the roles of sleep deprivation and/or selected drugs on locomotor activity, sedation, and nociception. During the experiments, 54 rats were found to have the correct microinjection sites; thus, their data were used for subsequent analysis.

3.2. Acute sleep deprivation increased expression of the Piezo1 protein in the basal forebrain

Western blot results showed a significant increase in Piezo1 protein expression in the BF after 6 h of sleep deprivation (t = 4.996, P < 0.01, Fig. 1B). Moreover, the TrkB-ICD (TrkB-FL degradation product) protein level was significantly increased in the BF after 6 h of sleep deprivation (t = 4.986, P < 0.01, Fig. 1C); however, no significant changes in total TrkB protein levels were observed after 6 h of sleep deprivation (t = 0.296, P > 0.05, Fig. 1D). Immunohistochemical staining showed that the number of Piezo1-positive cells in the BF was significantly increased in the sleep-deprived group compared with the control group (t = 5.827, P < 0.01, Fig. 1E–F).

3.3. Inhibition of Piezo1 signaling reduced TrkB degradation and counteracted acute sleep deprivation-induced fear memory impairments

We microinjected the selective Piezo1 inhibitor (GsMTx4) into the bilateral BF via the preplaced bilateral cannula to inhibit the Piezo1 pathway. Western blot results showed that GsMTx4 did not change the trend of Piezo1 protein expression (F(5,18) = 20.51, Fig. 2B) or TrkB phosphorylation (F(5,18) = 41.87, P < 0.01, Fig. 2C) induced by acute sleep deprivation; however, TrkB-ICD levels decreased after GsMTx4 microinjection (F(5,18) = 36.08, P < 0.01, Fig. 2D). GsMTx4 further enhanced the phosphorylation of PLCγ1, a downstream protein of TrkB activation, when combined with acute sleep deprivation (F(5,18) = 69.25, P < 0.01, Fig. 2E). In the step-down avoidance test, the latencies measured at 1 h and 24 h after training were significantly increased after sleep deprivation plus GsMTx4 microinjection (F(5,56) = 18.885, P < 0.01, Fig. 2F; F(5,56) = 21.000, P < 0.01, Fig. 2G, respectively) compared with sleep deprivation alone.

Microinjection of the selective Piezo1 activator Yoda1 into the BF mimicked the effects of sleep deprivation with increased TrkB-ICD levels (F(5,18) = 36.08, P < 0.01, Fig. 2D) and decreased PLCγ1 phosphorylation (F(5,18) = 69.25, P < 0.01, Fig. 2E) compared with the normal control. A significant synergistic effect on TrkB-ICD levels was observed when sleep deprivation and Yoda1 were concurrently administered to rats compared with sleep deprivation (P < 0.01) or Yoda1 alone (P = 0.031) (Fig. 2D). The Western blot results showed that Yoda1 did not change the trend of TrkB-ICD protein expression induced by sleep deprivation (F(5,18) = 20.51, Fig. 2B). In the step-down avoidance test, the latency measured at 1 h or 24 h after training was significantly shortened in sleep-deprived rats after Yoda1 microinjection (F(5,66) = 18.885, P < 0.01, Fig. 2F; F(5,66) = 21.000, P < 0.01, Fig. 2G, respectively).
Fig. 5. Summary of injection sites. (A) Excision position on the sagittal brain section of The Rat Brain In Stereotaxic Coordinates-5th edition (Paxinos & Watson, 2005). (B-G) Histological summary of all injection sites. The area depicted by the dotted line indicates the target injection site. The number of microinjections on and off the target area are also displayed. Only data from rats with correct bilateral injections were used in this study. A total of 282 rats were used, including 230 rats with correct bilateral microinjection, 42 rats with correct unilateral microinjection, and 10 rats with incorrect bilateral microinjection. (H) Representative image of the microinjection probe tip location. The probe track of the coronal brain section is shown on the left, which is consistent with a schematic drawing of the coronal brain section of The Rat Brain In Stereotaxic Coordinates-5th edition (Paxinos & Watson, 2005) on the right. Abbreviations: MCPO, magnocellular preoptic area; HDB, nucleus of the horizontal limb of the diagonal band; SI, substantia innominata; och, optic chiasm; 3v, 3rd ventricle; ac, anterior commissure; cc, corpus callosum; LV, lateral ventricle; B, basal nucleus (Meynert); VP, ventral pallidum; ic, internal capsule.
a family of Ca\textsuperscript{2+}-dependent nonlysosomal cysteine proteases [28], whose substrates include cytoskeletal proteins, N-methyl-D-aspartic acid receptor (NMDA) receptors, TrkB, and other proteins [29,30]. Studies have confirmed that overactivation of calpain triggers the formation of a truncated TrkB isoform (TrkB.T1) and an intracellular domain (TrkB-ICD) fragment, which contributes to the disruption of BDNF/TrkB signaling and cognitive impairments in mice [12,31]. Thus, inhibition of calpain-1 or calpain-2 in the hippocampus may represent a therapeutic treatment for some neurodegenerative disorders, such as seizure-induced neuropathology and AD [32–34]. In our previous study, we observed increased expression of the Piezo1 protein in the rat cerebral cortex after ischemia/reperfusion, which triggered an increased influx of calcium and subsequent overactivation of calcitonin [17,35]. Herein, we further proposed the hypothesis that the BF Piezo1/Ca\textsuperscript{2+}/calpain pathway modulates cleavage of TrkB-FL and impairments of fear encoding caused by sleep deprivation.

Several previous studies have shown that Ca\textsuperscript{2+} signaling is closely related to sleep [36–38]. Strong evidence has also emerged showing that calcium channel genes, such as CACNA1C (which encodes the L-type Ca\textsuperscript{2+} channel subunit), contribute to the affected phenotypes, such as memory and circadian rhythms [39,40]. Moreover, neuronal Ca\textsuperscript{2+} dysregulation plays an important role in AD pathogenesis [41]. Interestingly, L-type calcium channel blockers prevent cognitive deficits in some cognitive diseases, such as diabetic encephalopathy and AD, by attenuating increased calpain activity [41–43]. The Piezo1 ion channel is a new type of nonselective mechanically sensitive ion channel discovered in recent years. Clock genes regulate the circadian expression of Piezo1 [44], but the relationship between sleep deprivation and Piezo1 has not previously been reported. To the best of our knowledge, this is the first study to report that sleep deprivation (6 h) induced an increase in Piezo1 protein expression and TrkB-ICD expression in the BF (Fig. 1B–C). Furthermore, we observed that the activation of Piezo1 by Yoda 1 induces similar biological and memorial changes to sleep deprivation. TrkB-ICD expression was increased (Fig. 2D) and fear memory was impaired after microinjection of Yoda1 into the BF (Fig. 2F–G). More importantly, the inhibition of Piezo1 by GsMTx4 reduced the sleep deprivation-induced cleavage of TrkB (Fig. 2D) and activated the TrkB pathway (Fig. 2C and E). Meanwhile GsMTx4 partially reversed both the short- and long-term impairments of fear memory induced by acute sleep deprivation (Fig. 2F–G). Altogether, the results suggest that Piezo1 signaling plays a critical role in modulating cognitive impairments after sleep deprivation.

Activation of calpain is associated with Piezo1-mediated Ca\textsuperscript{2+} activities [17,45,46]. To investigate the mediating role of calpain signaling in the modulation of sleep deprivation-induced memory impairments by Piezo1, we used microinjected PD151746, a selective calpain inhibitor for calpain-1 and calpain-2, to the rat BF. We found that PD151746 reduced TrkB-ICD expression (Fig. 3C), activated the TrkB/Phospholipase C\gamma1 (PLC\gamma1) pathway (Fig. 3D and E), and partially reversed the short- and long-term impairments of fear memory encoding induced by acute sleep deprivation (Fig. 3F and G). TrkB-ICD is a stable protein fragment, which can accumulate in the nucleus for at least 8 h through a phosphorylation-dependent process [15]. Researchers have reported that TrkB-ICD contributes to a loss of BDNF signaling, whereas the overexpression of TrkB-FL improves spatial memory in mice [47,48]. In the present study, we also found that a decrease of TrkB-ICD expression by PIEZO1 or calpain inhibition using GsMTx4 or Yoda1 mitigated fear memory impairments caused by sleep deprivation (Figs. 2D, F and G, 3C, E, and 5C). Taken together, these results indicate the critical role of calpain-induced TrkB-FL cleavage in cognitive impairments after sleep deprivation.

We confirmed that sleep deprivation or drug injection had no effects on locomotor activity or sedation, but significantly lowered pain thresholds (Fig. 4). Previous research similarly found that total sleep deprivation impaired descending pain pathways, facilitated spinal excitability, and sensitized peripheral pathways to pressure pain in humans and animals [49,50]. BF cholinergic transmission may participate in the cerebral processing of pain, which may be relevant to the pain sensitivity through the medial forebrain bundle, which is a tract connecting the BF with the brain stem [51,52]. Changed pain sensation should have an effect on memory performance, as fear memory is closely related with electrical shock-induced foot pain. However, as sleep-deprived rats with lower pain thresholds experienced more intense pain, they should have more intense fear memory. Conversely, the fear memory results showed the opposite effect, with sleep deprivation causing substantial short and long-term fear memory impairments (Figs. 2F–G, 3F–G). Thus, changes in pain thresholds after sleep deprivation might not significantly change the nature of memory performance. Increasing evidence suggests that Piezo channels represent an attractive new molecular target to block pathological pain conditions via inhibition of membrane mechanotransduction [52]. In this study, we also found that inhibition of PIEZO1 by GsMTx4 after sleep deprivation increased the pain threshold (Fig. 4B).

Our data supports the proposed hypothesis that the BF Piezo1/Ca\textsuperscript{2+}/calpain pathway modulates the function of TrkB-FL and impairments of fear encoding caused by sleep deprivation; however, there are some limitations to our study that should not be ignored. Firstly, both Piezo1 and Piezo2 are essential components of distinct mechanically activated cation channels [35]. In this research, we predominantly observed the effect of Piezo1 expressed in the BF on sleep deprivation-induced fear memory impairments. Piezo2 is expressed in dorsal root ganglia neurons and Merkel cells as a sensor of light touch and proprioception [53,54]. However, researchers have not clearly determined whether Piezo2 is also expressed in the BF; the effect of Piezo2 on sleep deprivation-induced cognitive impairments will be examined in future studies. Secondly, calpain can be activated by Piezo1-induced calcium influx [45]. Although we found that 6 h of sleep deprivation increased Piezo1 expression, intracellular Ca\textsuperscript{2+} concentration and calpain activity were not investigated. Thirdly, calpains 1 and 2 share several common substrates, such as TrkB, which is the central receptor in the BDNF signaling pathway [30,55–57]. The calpain hyperactivation-induced cleavage of TrkB affects synaptic function and plasticity, which contribute to AD pathology [58]. Both calpain-1 and calpain-2 affect the induction and consolidation of long-term potentiation [59]. The major difference in calpain activation is that calpain-1 is activated by micro-molar concentrations of calcium, whereas calpain-2 is activated when calcium levels reach millimolar levels in vitro [60,61]. However, when calpain-2 is localized to the plasma membrane and interacts with phospholipids of the plasma membrane, it is activated by much lower intracellular calcium concentrations [62]. Calpain-1 hyperactivation, but not calpain-2 hyperactivation, is observed early in AD pathogenesis in mice and humans, whereas calpain-2 hyperactivation occurs later in the disease progression [63]. Thus, the roles of calpain-1 and calpain-2 in sleep deprivation will represent a future research direction. In addition, the selective antagonists GsMTx4 and PD151746 were delivered by microinjection in our study, but are not available for the treatment of human patients. Nevertheless, new drug vectors have been engineered and investigated for drug delivery and release, which might be used to activate Piezo1 through noninvasive methods. Stimuli-responsive polymeric nanocarriers can be engineered in different sizes, shapes, and surface charges for drug delivery across the blood brain barrier and in response to a specific stimulus, such as light, magnetism, ultrasound, or electrical pulses, thereby releasing the drug in the target brain region [64]. A magnetic toolkit has previously been used to stimulate Piezo1, which enabled consistent and reproducible neuromodulation in freely moving mice [65]. Therefore, these promising tools should be the focus of future research on sleep deprivation-induced cognitive impairments therapy.

5. Conclusion

In summary, acute total sleep deprivation for 6 h resulted in Piezo1/
Ca^{2+}/calpain signaling activation in the rat basal forebrain area, which cleaved the TrkB-FL receptor, leading to TrkB/PiGcR signaling dysfunction and fear memory impairments. The identification of viable therapeutic strategies to block the abnormal activation of Piezo1 or calpain in the basal forebrain may provide effective interventions for sleep deprivation-induced cognitive impairments.

CRediT authorship contribution statement

Tao Ma: Methodology, Investigation, Visualization, Writing – original draft. Ying-Ying Wang: Methodology, Visualization, Investigation, Resources. Yan Lu: Data curation, Methodology. Long Feng: Validation, Project administration. Yi-Tian Yang: Methodology, Data curation. Guan-Hua Li: Investigation, Resources. Chi Li: Supervision. Yang Chu: software validation. Hao Zhang: Conceptualization, Methodology, Writing – review & editing. Wei Wang: Conceptualization, Methodology, Writing – review & editing.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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