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Interplay between pain and cognitive dysfunction

Edited by
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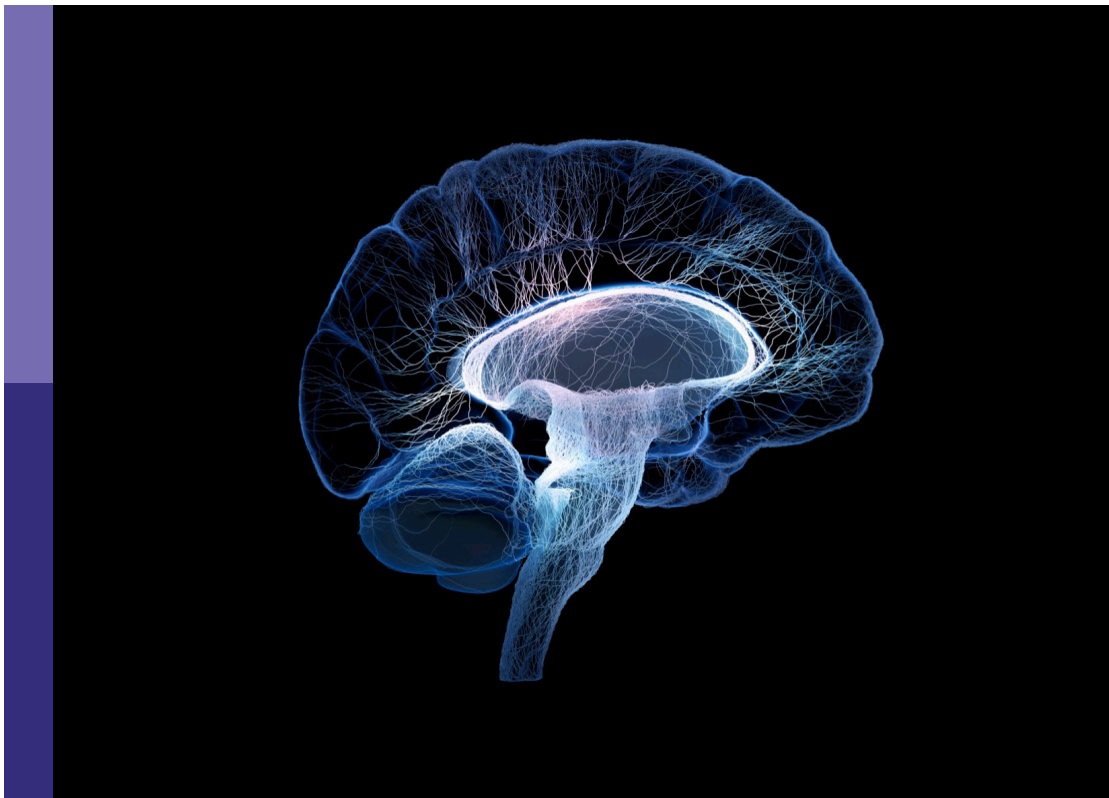

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Interplay between pain and cognitive dysfunction

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SYSTEMATIC REVIEW
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Evidence for Cognitive Decline in Chronic Pain: A Systematic Review and Meta-Analysis

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Background: People with chronic pain (CP) sometimes report impaired cognitive function, including a deficit of attention, memory, executive planning, and information processing. However, the association between CP and cognitive decline was still not clear. Our study aimed to assess the association of CP as a risk factor with cognitive decline among adults.

Methods: We included data from clinical studies. Publications were identified using a systematic search strategy from PubMed, Embase, and Cochrane Library databases from inception to October 10, 2020. We used the mean cognitive outcome data and the standard deviations from each group. The standardized mean difference (SMD) or odds ratio (OR), and 95% confidence intervals (CI) were performed for each cognitive decline outcome. I^2-values were assessed to quantify the heterogeneities.

Results: We included 37 studies with a total of 52,373 patients with CP and 80,434 healthy control participants. Because these studies used different evaluative methods, we analyzed these studies. The results showed CP was associated with cognitive decline when the short-form 36 health survey questionnaire (SF-36) mental component summary (SMD = −1.50, 95% CI = −2.19 to −0.81), the Montreal cognitive assessment (SMD = −1.11, 95% CI = −1.60 to −0.61), performance validity testing (SMD = 3.05, 95% CI = 1.74 to 4.37), or operation span (SMD = −1.83, 95% CI = −2.98 to −0.68) were used. However, we got opposite results when the studies using International Classification of Diseases and Related Health Problems classification (OR = 1.58, 95% CI = 0.97 to 2.56), the Mini-Mental State Examination (SMD = −0.42, 95% CI = −0.94 to 0.10; OR = 1.14, 95% CI = 0.91 to 1.42), and Repeatable Battery for the Assessment of Neuropsychological Status memory component (SMD = −0.06, 95% CI = −0.37 to 0.25).

Conclusion: There may be an association between CP and the incidence of cognitive decline when some cognitive, evaluative methods were used, such as short-form 36 health survey questionnaire, Montreal cognitive assessment, performance validity testing, and operation span.

Keywords: chronic pain, cognitive decline, memory, dementia, neuropathology

INTRODUCTION

Pain is a multidimensional experience that includes sensory discrimination, emotional motivation, and cognitive evaluation interacting with each other (Treede et al., 2019). And chronic pain (CP) refers to the persistent pain after healing, which is considered to have occurred or exist without tissue damage. Unfortunately, millions of people in today's world are debilitated by various CP types such as headaches, arthritis, and backache. Chronic pain is a common, complex, and distressing problem that has a major impact on society and individuals. The 2016 Global Burden of Disease Study highlighted that pain and

TABLE 1 | Characteristics of the included studies.

Study	Geographic location	Pain type	Participants					
			Chronic pain group			Healthy control group		
			N	Age	Gender	N	Age	Gender
Butterworth2014	Australia	FP	27	49.5 ± 8.7	22F	35	46.9 ± 8.8	27F
Coppieters2015	Belgium	WAD	16	41.6 ± 11.4	13F	22	38.0 ± 13.9	14F
		FM	21	44.5 ± 29.4	16f	22	38.0 ± 13.9	14F
Demirci2002	Turkey	headache	22	45.0 ± 8.6	21F	23	45 ± 8.6	21F
		LBP	23	47.6 ± 12	22F	23	45 ± 8.6	21F
Docking2014	UK	LBP	45	46.9 ± 11.9	34F	45	45.1 ± 10.4	34F
Dos Santos2016	Brasil	CP	45	N/A	N/A	45	N/A	N/A
Fernández2016	Spain	FP	22	47.9 ± 11.0	11F	22	47.2 ± 11	11F
Gu2019	China	CP	25	39.9 ± 9.9	11F	32	33.6 ± 8.7	9F
Hagen2014	Norway	headache	21,871	46.2 ± 15.2	14,063F	29,988	52.3 ± 17.7	13,944F
Ikram2019	USA	CP	6,379	N/A	N/A	12,504	N/A	N/A
Jonsson2011	Denmark	CP	62	52.2	35F	64	51.8	45F
Jordan2018	New Zealand	CP	5,287	82.48 ± 7.48	N/A	36,172	82.48 ± 7.48	N/A
Kaiho2017	Japan	CP	10,702	73.93 ± 5.86	4,730F	3,000	73.2 ± 6	1,858F
Ko2013	Korea	CAP	21	66.1 ± 11.4	8F	48	64.1 ± 13.1	10F
Kotb2020	Saudi Arabia	headache	100	35.31 ± 6.95	60F	105	35.51 ± 7.35	63F
Latysheva2020	Russia	headache	144	42.5 ± 3.17	132F	44	37 ± 5.5	40F
Martinsen2014	Sweden	FM	29	49.8 ± 9.75	N/A	31	46.3 ± 10.7	N/A
Meeks2008	USA	CP	92	79.4 ± 6.8	56F	56	81 ± 6.1	33F
Meeus2015	Belgium	WAD	15	41.6 ± 11.4	3F	16	40.9 ± 13.4	6F
Hirase2020	Japan	LBP,KP	421	75.79 ± 6.5	288F	368	73.65 ± 5.8	218F
Ojeda2017	Spain	NP,MP,FM	254	47.42 ± 8.8	N/A	72	40 ± 11.11	N/A
Öncü2015	Turkey	FM	86	32.3 ± 6.0	N/A	75	32.1 ± 7.7	N/A
Pickering2013	France	PHN	42	72 ± 8	N/A	42	72 ± 8	N/A
Pidal-Miranda2018	Spain	FM	38	47.71 ± 9.63	N/A	33	47 ± 9.01	N/A
Pirrotta 2013	Switzerland	NP	8	61.2 ± 13.2	N/A	9	60.6 ± 14.6	N/A
Qu2017	China	headache	51	37.6 ± 12.6	31F	28	33.9 ± 11.5	18F
Samartin2019	Spain	FM	18	43.9 ± 7.6	22F	22	45.1 ± 7.2	22F
Santangelo2016	Italy	headache	72	34.9 ± 11.2	63F	72	33.8 ± 11.9	66F
Schepker2016	USA	CP	146	75.5 ± 7.23	108F	284	77.1 ± 16.84	183F
Shega2010	Canada	Non-CAP	1,813	79.9 ± 6.0	1,222F	3,273	79.4 ± 6.1	1,817F
Shega2012	Canada	CP	1,332	79.4 ± 5.8	922F	2,435	78.9 ± 5.8	1,359F
Tzeng2017	Taiwan	headache	3,630	N/A	2,463F	10,860	N/A	7,389F
vander Leeuw2018	USA	CP	285	N/A	N/A	156	N/A	N/A
vander Leeuw2019	USA	CP	692	74.38 ± 6.59	480F	2,552	74.29 ± 6.81	1,430F
Veronese2018	Italy	CP	2,317	65.5 ± 9.5	1,244F	4,198	64.2 ± 9.7	2,674F
Walteros2011	Spain	FM	15	50.4 ± 4.6	N/A	15	49.0 ± 6.7	N/A
Weiner2006	USA	LBP	163	73.6 ± 5.2	80F	160	73.5 ± 4.8	66F
Whitlock 2017	USA	CP	1,120	73.8 ± 5.4	851F	8,945	73.6 ± 5.2	5,197F

FP, foot pain; WAD, Whiplash-associated disorder; FM, Fibromyalgia syndrome; LBP, low back pain; CP, chronic pain; CAP, cancer pain; KP, knee pain; NP, neuropathic pain; MP, musculoskeletal pain; PHN, postherpetic neuralgia; UK, United Kingdom; USA, United States of America; N, number; F, female; N/A, not applicable.

pain-related diseases were major contributors to the global burden of disability and disease (GBD Disease and Injury Incidence and Prevalence Collaborators, 2017). The annual costs of CP in direct medical costs are very high, and the loss of productivity it brings is huge. Therefore, pain relief is an important topic in clinical work and scientific research. Moreover, increased studies have shown that CP had many adverse outcomes, such as mood disorder, weight loss, daily functional loss, lower quality of life, and higher costs of health (Saraiva et al., 2018). Interestingly, CP seems to cause changes in cognitive function, which is another critical problem.

Cognitive decline could involve cognitive impairment in one or several areas, such as learning and memory, executive function, general cognitive functioning, attention, and social cognition (Sachdev et al., 2014). Moreover, cognitive decline has been reported to significantly impact medical compliance, workability, interpersonal interaction, and quality of life. Clinical studies have shown that CP was related to attention, memory, executive planning, and information processing (Berryman et al., 2013, 2014; Mazza et al., 2018). A longitudinal study of elders found persistent pain was associated with memory decline (Whitlock et al., 2017). A meta-analysis of longitudinal studies showed that headache was associated with a higher risk of dementia (Wang et al., 2018). However, another meta-analysis contained 10 prospective longitudinal studies that showed persistent pain was not associated with the incidence of cognitive decline (De Aguiar et al., 2020). A study also demonstrated pain was not associated with incident cognitive impairment in any of the three cognitive domains evaluated (attention, memory, and executive functioning) (Van Der Leeuw et al., 2018a).

The relation between pain and cognitive decline is complex, and pain may impair cognition by some mechanisms (Moriarty et al., 2011). Some studies suggested that the pain was associated with brain plasticity and structural changes in different cortical regions associated with learning, memory, fear, and emotional responses (Mazza et al., 2018). In addition, Moriarty et al. (2011) demonstrated that pain and cognition might share some common transduction pathways. Because pain is treatable, defining whether it is a risk factor of cognitive decline can promote targeted screening, preventive, and therapeutic interventions. Therefore, we applied a systematic review and meta-analysis to determine the evidence that CP is associated with cognitive decline.

METHODS

Search Strategy

We searched the PubMed, Embase, and Cochrane Library from inception to October 10, 2020. Each database was searched separately. We searched synonyms of "chronic pain" and "cognitive decline." Synonyms for "chronic pain" included "Chronic Pains," "Pains, Chronic," "Pain, Chronic," "Widespread Chronic Pain," "Chronic Pain, Widespread," "Chronic Pains, Widespread," "Pain, Widespread Chronic," "Pains, Widespread Chronic," and "Widespread Chronic Pains." Synonyms for "Cognitive Decline" included "Cognitive Dysfunctions," "Dysfunction, Cognitive," "Dysfunctions, Cognitive," "Cognitive Impairments," "Cognitive Impairment," "Impairment, Cognitive," "Impairments, Cognitive," "Mild Cognitive Impairment," "Cognitive Impairment, Mild," "Cognitive Impairments, Mild," "Impairment, Mild Cognitive," "Impairments, Mild Cognitive," "Mild Cognitive Impairments," "Mild Neurocognitive Disorder," "Disorder, Mild Neurocognitive," "Disorders, Mild Neurocognitive," "Mild Neurocognitive Disorders," "Neurocognitive Disorder, Mild," "Neurocognitive Disorders, Mild," "Cognitive Decline," "Cognitive Declines," "Decline, Cognitive," "Declines, Cognitive," "Mental Deterioration," "Deterioration, Mental," "Deteriorations, Mental," "Mental Deteriorations," "Cognitive disorders," "Disorder, Cognition," "Disorders, Cognition," "Dementia," and "Dementias." Citations related to cognitive decline and CP were retrieved and exported to ENDNOTE, where duplicates were removed, and articles were reviewed. This systematic review and meta-analysis were followed according to Cochrane Collaboration (Higgins and Green, 2008) and Preferred Reporting Items for Systematic Reviews and Meta-Analyses Statement guidelines (Moher et al., 2009). The authors have completed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses reporting checklist (**Supplementary Material**).

Study Selection

In this meta-analysis, we included studies that investigated cognitive performance in a CP population and compared this performance with that of healthy controls. Two coauthors (Xueying Zhang and Qi Zhao) independently reviewed the titles and abstracts of the retrieved citations. First, we excluded studies that were not investigating the association of pain and cognitive decline. The discrepancy between these two coauthors was reviewed by another author (Rui Gao). Then, full texts of

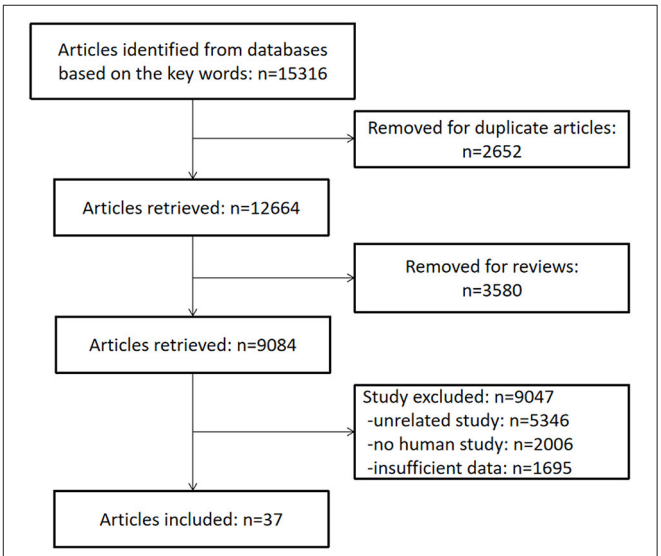

FIGURE 1 | Flow diagram of the literature search.

TABLE 2 | Risk of bias of included studies.

Study (ref.)	Are cases represe-ntative?	Were initial numbers recorded?	Were cases diagnosed according to accepted criteria?	Were controls using the same diagnostic criteria?	Were psychiatric disorders screened for?	Were outcome assessors blinded to group status?	Were sample sizes calculated a priori?	Were confounding variables controlled for?	Was subgroup evaluation appropriate?	Were there any missing data?	Appropriate methods to deal with missing data?	Were all outcomes and groups reported?	Are the cognitive tests used valid?	Are the cognitive tests used reliable?
Butterworth2014	Y	Y	Y	Y	N	?	N	Y	Y	Y	Y	Y	Y	Y
Coppieters2015	Y	Y	Y	Y	Y	?	N	Y	Y	N	N	Y	Y	Y
Demirci2002	Y	Y	Y	Y	Y	?	N	Y	Y	Y	Y	Y	Y	Y
Docking2014	Y	Y	N	Y	Y	?	N	Y	Y	Y	Y	Y	Y	Y
Dos Santos2016	Y	Y	Y	Y	Y	?	N	Y	Y	Y	Y	Y	Y	Y
Fernández2016	Y	Y	Y	Y	N	?	N	Y	Y	N	N	Y	Y	Y
Gu2018	Y	Y	Y	Y	Y	?	N	Y	Y	Y	Y	Y	Y	Y
Hagen2013	Y	Y	Y	Y	N	?	N	Y	Y	Y	Y	Y	Y	Y
Ikram2019	Y	Y	Y	Y	Y	?	Y	Y	Y	N	N	Y	Y	Y
Jonsson2011	Y	Y	Y	Y	N	?	N	Y	Y	Y	Y	Y	Y	Y
Jordan2018	Y	Y	Y	Y	N	?	N	Y	Y	Y	Y	Y	Y	Y
Kaiho2017	Y	Y	Y	Y	N	?	N	Y	Y	Y	Y	Y	Y	Y
Ko2013	Y	Y	Y	Y	Y	?	N	Y	Y	N	N	Y	Y	Y
Kotb2020	Y	Y	Y	Y	Y	?	N	Y	Y	N	N	Y	Y	Y
Latysheva2020	Y	Y	Y	Y	Y	?	N	Y	Y	N	N	Y	Y	Y
Martinsen2014	Y	Y	Y	Y	Y	?	N	Y	Y	N	N	Y	Y	Y
Meeks2008	Y	Y	N	Y	Y	?	N	Y	Y	Y	Y	Y	Y	Y
Meeus2015	Y	Y	Y	Y	Y	?	N	Y	Y	N	N	Y	Y	Y
Hirase2020	Y	Y	N	Y	Y	?	N	Y	Y	N	N	Y	Y	Y
Ojeda2017	Y	Y	Y	Y	Y	?	N	Y	Y	N	N	Y	Y	Y
Öncü2015	Y	Y	Y	Y	Y	?	N	Y	Y	N	N	Y	Y	Y
Pickering2013	Y	Y	Y	Y	Y	?	N	Y	Y	N	N	Y	Y	Y
Pidal-Miranda2018	Y	Y	Y	Y	Y	?	N	Y	Y	N	N	Y	Y	Y
Pirrotta 2013	Y	Y	Y	Y	Y	?	Y	Y	Y	Y	Y	Y	Y	Y
Qu2017	Y	Y	Y	Y	Y	?	N	Y	Y	N	N	Y	Y	Y
Samartin2019	Y	Y	Y	Y	Y	?	N	Y	Y	N	N	Y	Y	Y
Santangelo2016	Y	Y	Y	Y	Y	?	N	Y	Y	N	N	Y	Y	Y
Schepker2016	Y	Y	Y	Y	Y	?	N	Y	Y	N	N	Y	Y	Y
Shega2010	Y	Y	Y	Y	Y	?	N	Y	Y	N	N	Y	Y	Y
Shega2012	Y	Y	Y	Y	Y	?	N	Y	Y	N	N	Y	Y	Y
Tzeng2017	Y	Y	Y	Y	Y	?	N	Y	Y	Y	Y	Y	Y	Y

(Continued)

TABLE 2 | Continued

Study (ref.)	Are cases representative?	Were initial numbers recorded?	Were cases diagnosed according to accepted criteria?	Were controls using the same diagnostic criteria?	Were psychiatric disorders screened for?	Were outcome assessors blinded to group status?	Were sample sizes calculated a priori?	Were confounding variables controlled for?	Was subgroup evaluation appropriate?	Were there any missing data?	Appropriate methods to deal with missing data?	Were all outcomes and groups reported?	Are the cognitive tests used valid?	Are the cognitive tests used reliable?
vander Leeuw2018	Y	Y	Y	Y	Y	?	N	Y	Y	Y	Y	Y	Y	Y
vander Leeuw2019	Y	Y	Y	Y	Y	?	N	Y	Y	Y	Y	Y	Y	Y
Veronese2018	Y	Y	N	Y	Y	?	N	Y	Y	Y	Y	Y	Y	Y
Walteros2011	Y	Y	Y	Y	Y	?	N	Y	Y	Y	Y	Y	Y	Y
Weiner2006	Y	Y	N	Y	Y	?	N	Y	Y	Y	Y	Y	Y	Y
Whitlock 2017	Y	Y	Y	Y	Y	?	N	Y	Y	Y	Y	Y	Y	Y

Y, Yes; N, No; ?, unknown.

the selected citations were assessed independently for inclusion criteria by the same coauthors and using the same strategy.

We included randomized clinical trials, systematic reviews, and meta-analyses. We excluded editorials, case reports, and descriptive and cross-sectional studies. The definition of cognitive decline could be reported by incident cognitive impairment (binary outcomes) or a decline in cognitive performance (continuous outcomes). For pain assessment, we included studies reporting any kind of pain, except experimental pain.

Data Extraction

Two coauthors (Xueying Zhang and Changteng Zhang) independently read and extracted data from the included full-text citations. For each study included, we extracted this information: study publication year, geographic location where the study was performed, numbers, type of pain, age, and sex distribution of each group, mean age of participants, and number of female participants.

Risk of Bias Assessment

We constructed a risk of bias form based on relevant items from the Cochrane Collaboration risk of bias tool and relevant forms of bias relating to case–control study designs (Berryman et al., 2013). Two coauthors (Xueying Zhang and Qi Zhao) completed the bias of each study. Any disagreements were resolved through discussion or by the inclusion of a third reviewer (Rui Gao).

Data Analysis

Cognitive outcome data were divided according to different cognitive diagnosis methods. We used the mean cognitive outcome data and the standard deviations from each group. The standardized mean difference (SMD) of continuous data or odds ratio (OR) of binary data and 95% confidence intervals (CI) was performed for each cognitive decline outcome. I^2-values were assessed to quantify the heterogeneities. Data for each cognitive decline construct were pooled when results were available from at least two studies. Studies were excluded from the analysis if they did not have sufficient data.

RESULTS

Characteristics of the Included Studies

From the 15,316 records identified by the search methods, 37 studies (Demirci and Savas, 2002; Weiner et al., 2006; Meeks et al., 2008; Shega et al., 2010, 2012; Jonsson et al., 2011; Walteros et al., 2011; Ko et al., 2013; Pirrotta et al., 2013; Butterworth et al., 2014; Docking et al., 2014; Hagen et al., 2014; Martinsen et al., 2014; Pickering et al., 2014; Coppieters et al., 2015; Meeus et al., 2015; Öncü et al., 2015; Dos Santos Ferreira et al., 2016; Fernández-Lao et al., 2016; Santangelo et al., 2016; Schepker et al., 2016; Kaiho et al., 2017; Tzeng et al., 2017; Whitlock et al., 2017; Jordan et al., 2018; Ojeda et al., 2018; Pidal-Miranda et al., 2018; Qu et al., 2018; Van Der Leeuw et al., 2018b, 2020; Veronese et al., 2018; Gu et al., 2019; Ikram et al., 2019; Samartin-Veiga et al., 2019; Kotb et al., 2020; Latysheva

et al., 2020; Nakai et al., 2020) were identified, a total of 52,373 patients with CP and 80,434 healthy control participants. These 37 studies included different types of CP: headache ($n = 7$); fibromyalgia syndrome (FM) ($n = 6$); low back pain ($n = 3$); whiplash-associated disorder ($n = 2$); foot pain ($n = 2$); cancer pain ($n = 1$); low back pain and knee pain ($n = 1$); neuropathic pain ($n = 1$); musculoskeletal pain, neuropathic pain, and FM ($n = 1$); post-herpetic neuralgia ($n = 1$); non-cancer pain ($n = 1$); and other unclassified CP ($n = 13$). Among these 37 studies, there were two studies that have invested two kinds of CP, so the total number is 39. Of these 37 studies, cognitive decline was evaluated by different methods: Mini-Mental State Examination (MMSE) ($n = 6$), the short-form 36 health survey questionnaire (SF-36) mental component ($n = 6$), Montreal cognitive assessment (MOCA) ($n = 4$), International Classification of Diseases and Related Health Problems (ICD) classification ($n = 4$), Wechsler Adult Intelligence Scale (WAIS) ($n = 4$), performance validity testing (PVT) ($n = 2$), operation span (OSPAN) ($n = 2$), Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) memory component ($n = 2$) (**Table 1**), Stroop test ($n = 1$), Memory Failures of Everyday (MFE-30) test ($n = 1$), Syndrom Kurz Test (SKT) ($n = 1$), Cognitive Performance Scale minimus data set (MDS) ($n = 1$), Telephone Interview for Cognitive Status (TICS) ($n = 1$), short test of mental status (STMS) ($n = 1$), and Cambridge Neuropsychological Test Automated Battery (CNTAB) ($n = 1$). **Figure 1** describes the process of the study selection. **Table 1** describes the characteristics of the included studies.

Only when at least two articles used the same method can be used for meta-analysis. Also, as for WAIS, the four studies used different parts of WAIS, so they could not be analyzed together. Furthermore, for SF-36, mental health and mental component summary are considered as two comparisons. Effects were expressed as SMD for continuous data and OR for binary data, and 95% CIs were given for each study. Weights are from the random-effects analysis.

Risk of Bias of Included Studies

All studies were deemed to have a risk of bias. One hand is owing to the lack of blinding of the outcome assessors and patients. On the other hand, the bias of these studies was mainly in whether psychiatric disorders were screened for, sample sizes calculated *a priori* (**Table 2**).

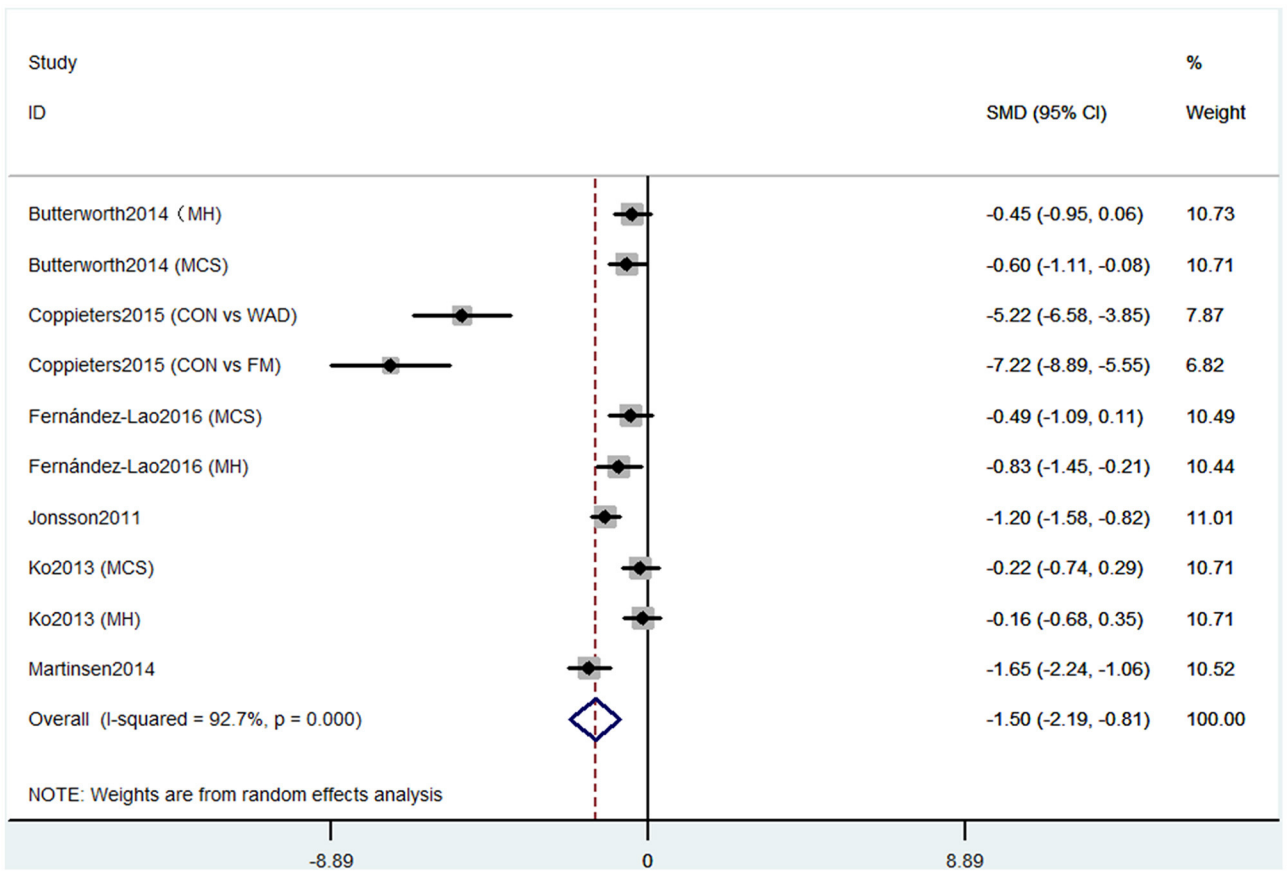

FIGURE 2 | Forest plot of the SMD and 95% CI from a random-effects meta-analysis for the association between chronic pain and incidence of cognitive decline when SF-36 mental component summary was used. MH, mental health score of SF-36; MCS, mental component summary score of sf-36; CON, control group; WAD, whiplash-associated disorder group; FM, fibromyalgia syndrome group.

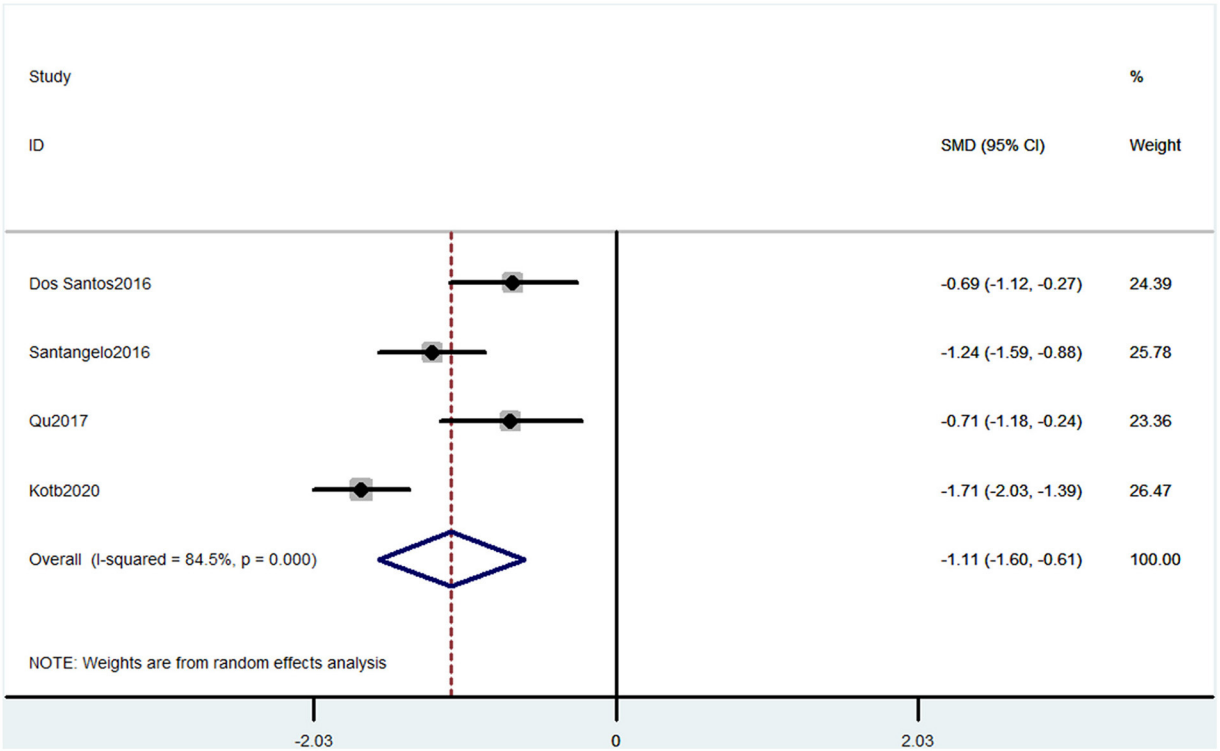

FIGURE 3 | Forest plot of the SMD and 95% CI from a random-effects meta-analysis for the association between chronic pain and incidence of cognitive decline when MOCA was used. The rhombi represent the pooled SMD for this association.

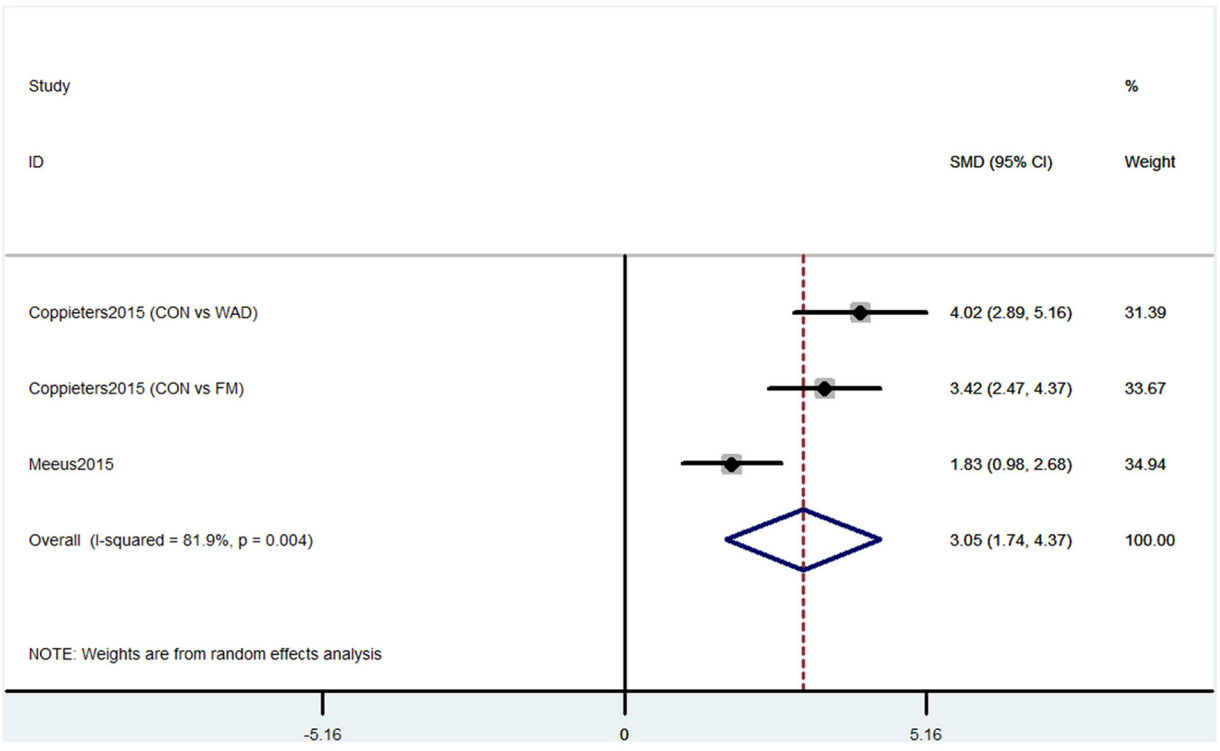

FIGURE 4 | Forest plot of the SMD and 95% CI from a random-effects meta-analysis for the association between chronic pain and incidence of cognitive decline when PVT was used. The rhombi represent the pooled SMD for this association. CON, control group; WAD, whiplash-associated disorder group; FM, fibromyalgia syndrome group.

How Cognitive Decline Was Evaluated—Tests and Test Outcomes

Among the 37 included studies, four studies used ICD to identify the cognitive decline diseases, six studies contained 10 comparisons that used SF-36 mental component summary, six studies contained nine comparisons that used MMSE, and four studies used MOCA. In addition, two studies contained three comparisons that used PVT, two studies contained three comparisons that used OSPAN, and two studies used RBANS memory component to evaluate cognition function. Besides these, which can be analyzed, there were 11 studies that used other methods, which could not be analyzed. Four studies contained four comparisons that used different parts of the WAIS scale. Other studies used the Stroop test, MFE-30, SKT, MDS, TICS, STMS, and CNTAB, each contained one comparison.

Meta-Analysis Outcomes

Outcome 1: Results for Short-Form 36 Health Survey Questionnaire Mental Component

Short-form 36 health survey questionnaire was used by six studies containing 10 comparisons to evaluate the cognitive decline that mainly refers to mental health. Pooled results of these studies showed that CP was associated with cognitive decline (SMD = −1.50, 95% CI = −2.19 to −0.81). The patients in the pain group were more likely to get the mental problem (**Figure 2**).

Outcome 2: Results for Montreal Cognitive Assessment

Four studies used MOCA to evaluate cognitive decline. The pooled results of these studies showed that CP was associated with cognitive decline (SMD = −1.11, 95% CI = −1.60 to −0.61). The patients in the pain group were more likely to get low MOCA scores (**Figure 3**).

Outcome 3: Results for Performance Validity Testing

Performance validity testing was used by two studies containing three comparisons to evaluate the cognitive decline. The PVT has been validated as a measure of sustained attention, alertness, and simple reaction time. The pooled results showed that CP was associated with cognitive decline (SMD = 3.05, 95% CI = 1.74 to 4.37). The patients in the pain group were more likely to have poor cognitive performance (**Figure 4**).

Outcome 4: Results for Operation Span

Operation span was used by two studies containing three comparisons to evaluate the cognitive decline. This task was used to assess working memory capacity. The pooled results showed that CP was associated with cognitive decline (SMD = −1.83,

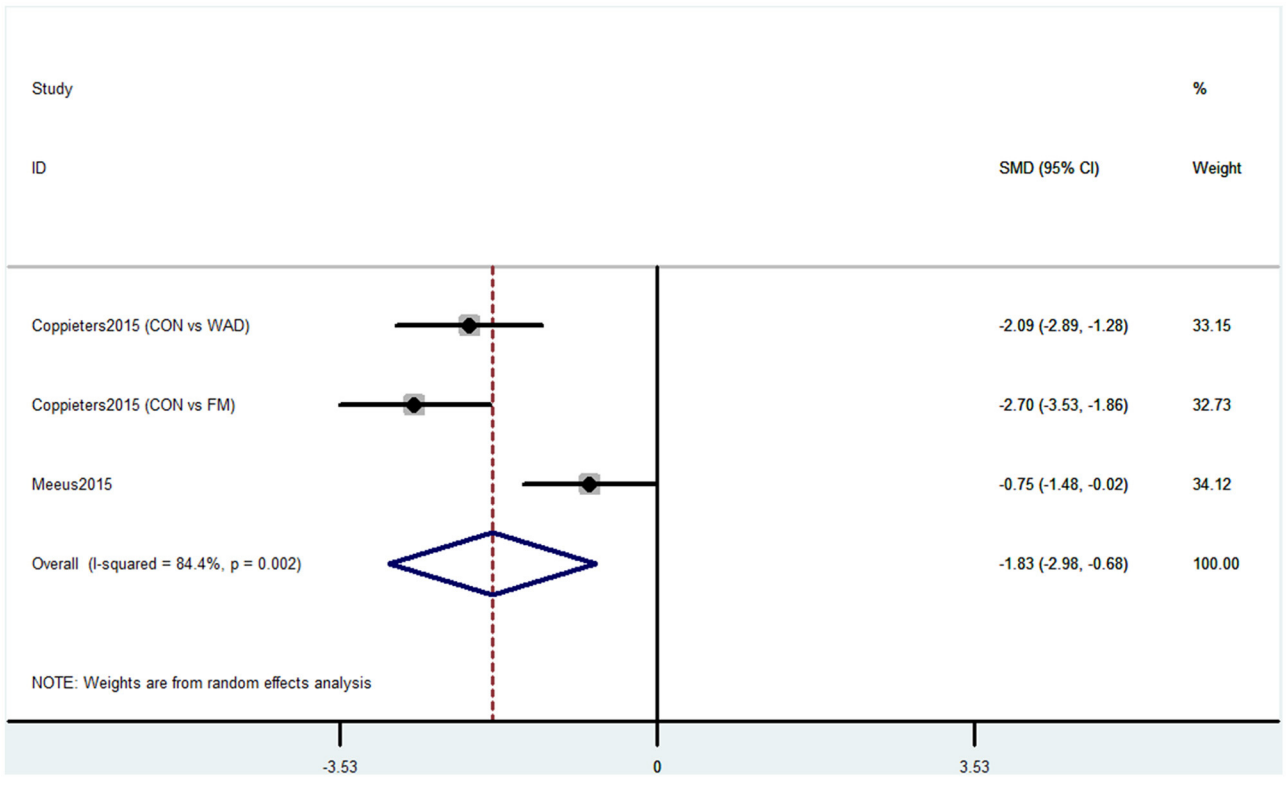

FIGURE 5 | Forest plot of the SMD and 95% CI from a random-effects meta-analysis for the association between chronic pain and incidence of cognitive decline when OSPAN was used. The rhombi represent the pooled SMD for this association. CON, control group; WAD, whiplash-associated disorder group; FM, fibromyalgia syndrome group.

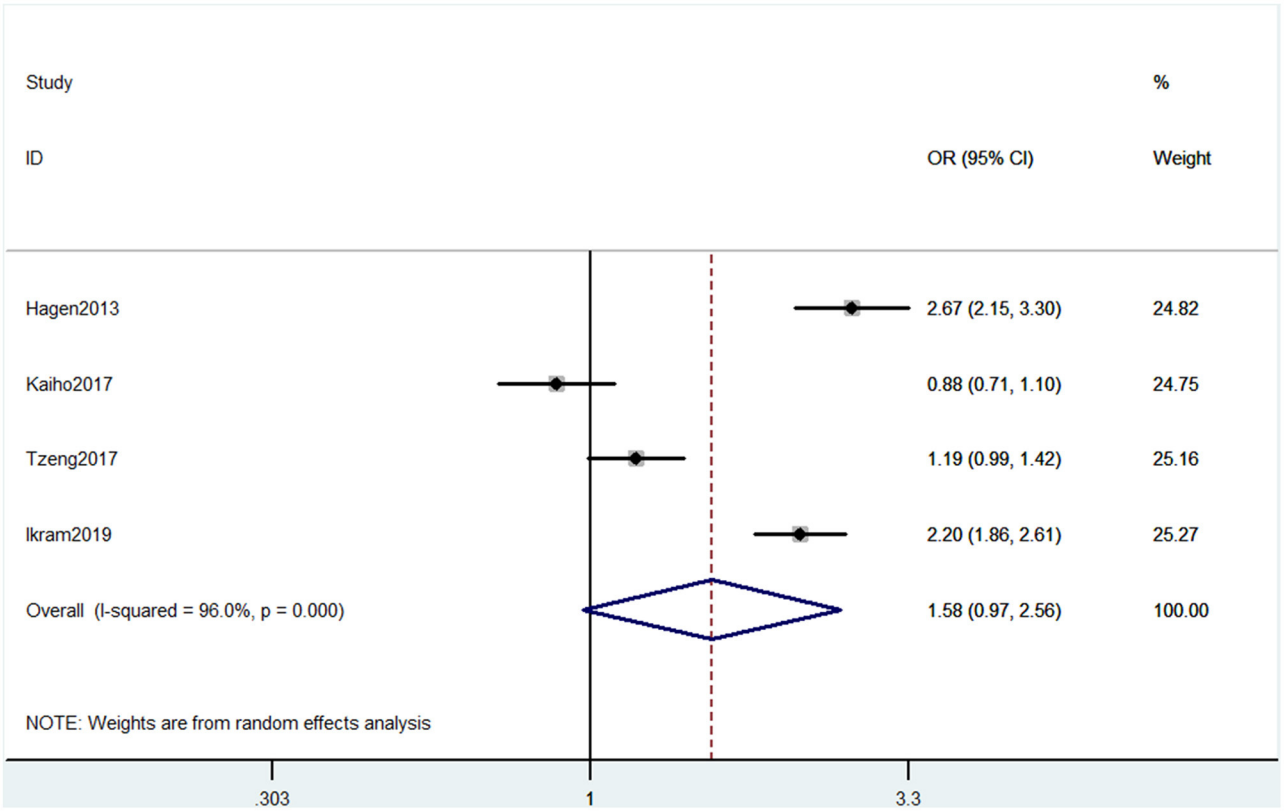

FIGURE 6 | Forest plot of the OR and 95% CI from a random-effects meta-analysis for the association between chronic pain and incidence of cognitive decline when ICD was used. The rhombi represent the pooled OR for this association.

95% CI = −2.98 to −0.68). The patients in the pain group were more likely to have poor working memory performance (**Figure 5**).

Outcome 5: Results for International Classification of Diseases and Related Health Problems

Four studies used ICD to evaluate cognitive decline. The types of cognitive decline defined in these four studies mainly contained dementia and Alzheimer's disease. Pooled results of these studies showed that CP was not associated with cognitive decline (OR = 1.58, 95% CI = 0.97 to 2.56) (**Figure 6**).

Outcome 6: Results for Mini-Mental State Examination

Six studies used MMSE to evaluate cognitive decline. Among these six studies, four studies containing five comparisons used MMSE primary scores as the results. The pooled results showed that CP is not associated with cognitive decline (SMD = −0.42, 95% CI = −0.94 to 0.10) (**Figure 7**). Also, three studies contained four comparisons that divided participants into normal cognition and impaired cognition due to the scores. The pooled studies also showed that CP was not associated with cognitive decline (OR = 1.14, 95% CI = 0.91 to 1.42) (**Figure 8**).

Outcomes 7: Results for Repeatable Battery for the Assessment of Neuropsychological Status

Two studies used the RBANS memory component to evaluate cognitive decline. The pooled results showed that CP was not associated with cognitive decline (SMD = −0.06, 95% CI = −0.37 to 0.25) (**Figure 9**).

DISCUSSION

Cognition is a complex concept that includes learning, memory, language, executive function, attention, social cognition, and others (Sachdev et al., 2014). The evaluative method of cognition remains various, making it difficult for researchers to conduct clinical trials or meta-analyses. We performed the analysis due to the different methods for cognitive assessment used in the 37 included articles. Pooled results from the outcomes of our study reflect an opposite result. The results showed a consistent, significant effect between CP and cognitive decline when the methods of SF-36 mental component summary, MOCA, PVT, and OSPAN were used. However, the results provided no evidence for an effect when methods of ICD, MMSE, and RBANS were used.

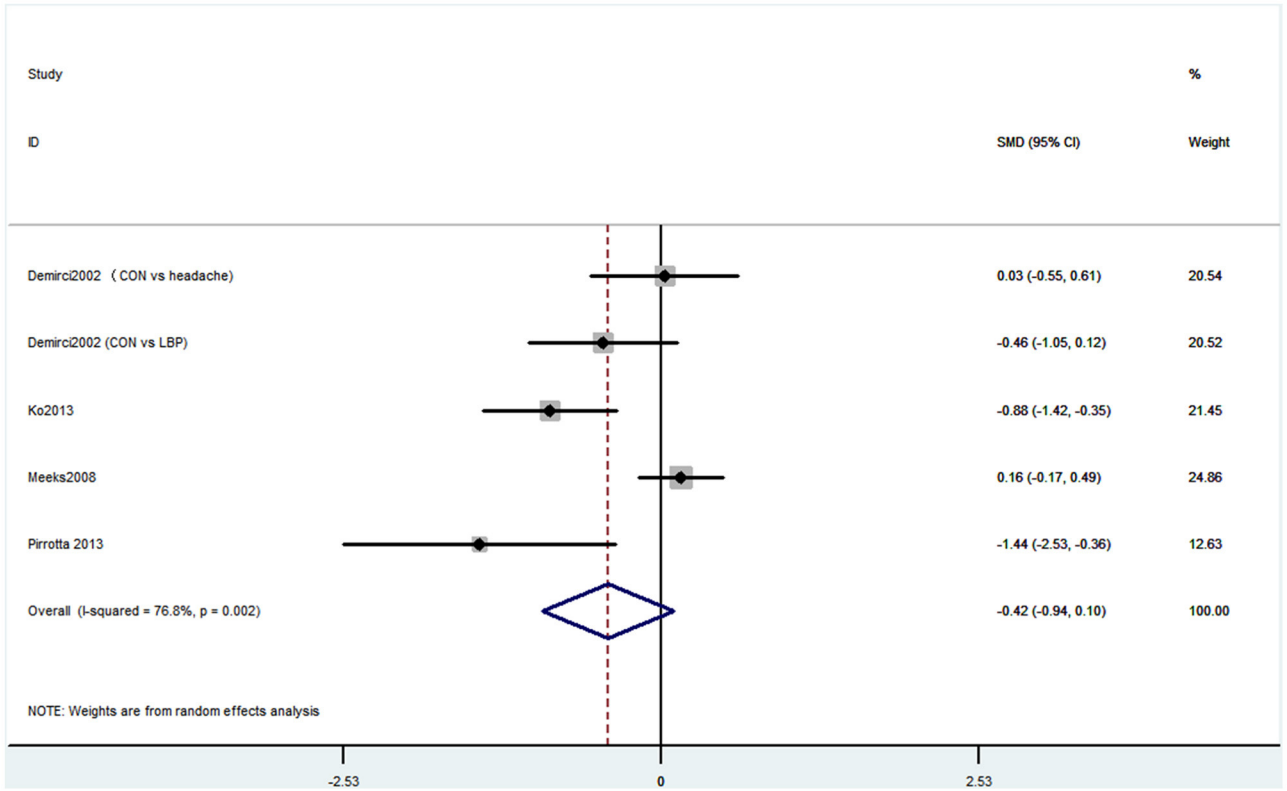

FIGURE 7 | Forest plot of the SMD and 95% CI from a random-effects meta-analysis for the association between chronic pain and incidence of cognitive decline when MMSE continuous score was used. The rhombi represent the pooled SMD for this association. CON, control group; LBP, low back pain.

Numerous studies containing clinical and animal studies have shown CP could result in cognitive decline (Moriarty et al., 2011; Yang et al., 2014; Whitlock et al., 2017; Shiers et al., 2020). For example, Whitlock et al. (2017) found persistent pain was associated with accelerated memory decline and increased probability of dementia based on a cohort study with biennial interviews of 10,065 community-dwelling older adults. This article was published in the *Journal of the American Medical Association Internal Medicine*. Mechanism studies explain pain, and cognitive decline may share some same pathways (Moriarty et al., 2011; Phelps et al., 2021). Also, there have been some meta-analyses exploring the relationship between pain and some types of cognitive decline. For example, one meta-analysis showed CP was associated with memory deficits but only in behavioral outcomes, whereas the physiological effects showed no effect (Berryman et al., 2013). Another meta-analysis focused on executive function showed that people with CP might have impaired executive function (Berryman et al., 2014). However, a recent meta-analysis showed persistent pain was not associated with the incidence of cognitive decline from prospective longitudinal studies (De Aguiar et al., 2020).

Our meta-analysis showed some evidence that CP might be related to cognitive impairment. Six studies containing 10 comparisons used SF-36 that show that CP was associated with cognitive decline. The SF-36 was used to assess physical function, mental health, and health-related quality of life (Brazier et al., 1992). These six studies were from different countries, and the heterogeneity was high. Meanwhile, the pain types of these studies were different, four studies were CP without any subgroups, one study contained both whiplash-associated disorder and FM (Coppieters et al., 2015), and one study only contained FM (Martinsen et al., 2014). These factors together may induce high heterogeneity. Studies using MOCA also showed that CP was associated with cognitive decline. MOCA is used to evaluate the global cognitive status and several cognitive domains: memory, attention, language, orientation, and visuospatial and executive function domains (Nasreddine et al., 2005). These studies also had high heterogeneity. Three studies were performed on headache patients, but one investigated CP without more detailed classification. Thus, more homogeneous controlled clinical trials are needed to confirm the results. The PVT has been validated as a measure of sustained attention, alertness, and simple reaction time. Two studies containing three comparisons used PVT also showed that CP was associated with cognitive decline. However, only two studies were included in this analysis. Therefore, the number of included participants may not be enough, and more studies are needed. Another evidence was that two studies using OSPAN to

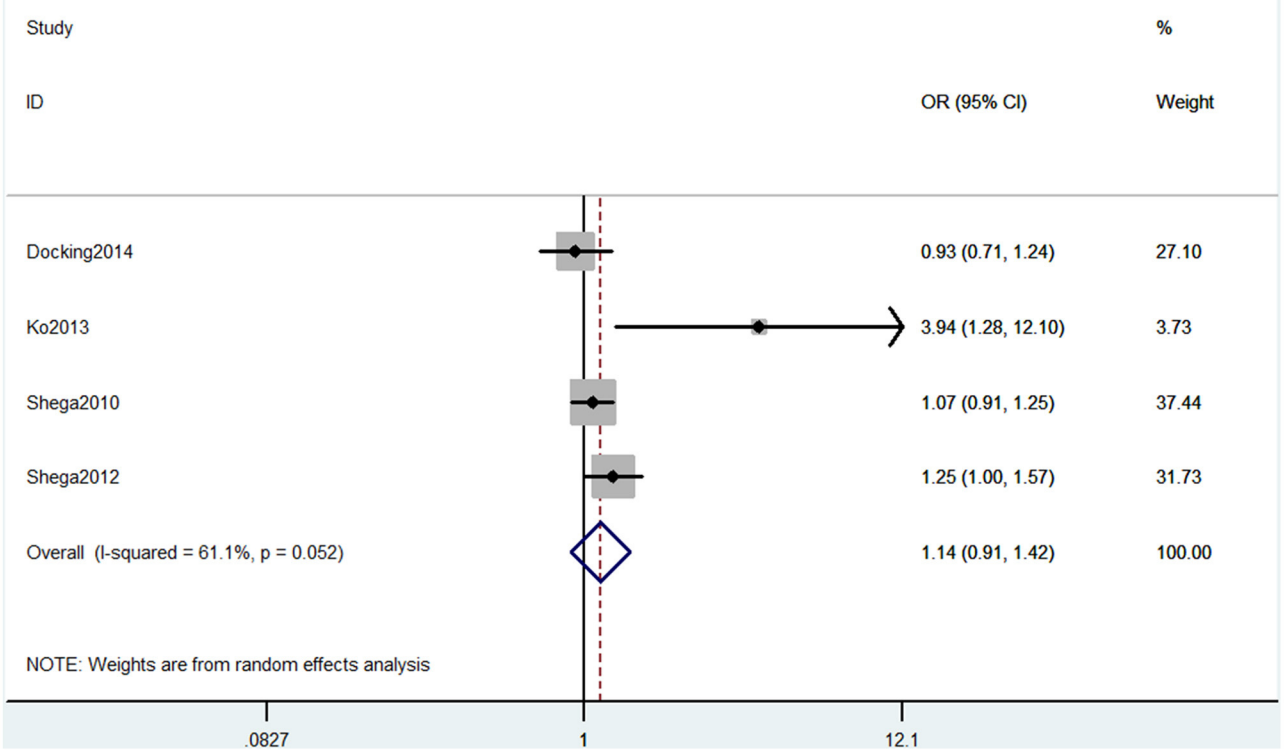

FIGURE 8 | Forest plot of the OR and 95% CI from a random-effects meta-analysis for the association between chronic pain and incidence of cognitive decline when MMSE binary method was used. The rhombi represent the pooled OR for this association.

evaluate memory also verified the association between CP and cognitive decline (Quach et al., 2016). Besides, a study that used SKT also showed that preoperative CP distracted the attention before surgery and reduced the recovery of attention and memory abilities after the surgery in non-elderly patients (Gu et al., 2019). Moreover, animal studies showed that both rats and mice with CP could have cognitive disorders (Owoyele et al., 2021; Zhang et al., 2021).

The association between CP and cognitive decline is still unknown. An important key is that it is difficult to combine all the evaluative methods of cognition. In some methods such as MMSE, the higher the score, the worse the cognitive impairment. Some others, such as SF36, are opposite. In addition, some methods studied different types of cognitive impairment and could not be analyzed together. A total of 37 articles included in our study contained 15 methods that have been listed earlier. The sample size of studies using the same method was different. The study of Hagen et al. (2014) has the most sample size and far exceeded other studies. Another study with a larger sample size is Kaiho et al. (2017). Furthermore, these two studies both used the ICD method. However, the negative result of Kaiho et al. (2017) reversed the positive results of Hagen et al. (2014) and the other two studies in outcome 5 (**Figure 6**). Also, for other outcomes, whether the sample size is enough to support the positive results is unsure. So the conclusion needs a larger sample size and multicenter studies. Secondly, the evaluative accuracy of these methods is also different. Mini-Mental State Examination and MOCA, as the most basic methods, are regarded as brief screening tools (Nasreddine et al., 2005; Ciesielska et al., 2016). Wechsler Adult Intelligence Scale is also a comprehensive scale for neuropsychological status, including reasoning, processing speed, and working memory (Whipple Drozdick and Munro Cullum, 2011). Short-form 36 health survey questionnaire is a health-related evaluation tool, so the mental component summary and mental health part could only reflect some mental health (Brazier et al., 1992). Short test of mental status was found to be more sensitive than MMSE and can be used by clinicians to differentiate both normal cognition from MCI and MCI from probable Alzheimer's disease (Çebi et al., 2020). Performance validity testing, OSPAN, RBANS, and SKT are used to evaluate some aspects, including memory, attention, and reaction (Meeus et al., 2015; Van Der Leeuw et al., 2018a; Gu et al., 2019; Loring and Goldstein, 2019). Stroop test is used to mainly test reactions (Coppieters et al., 2015). Memory Failures of Everyday is used to assess memory problems (Samartin-Veiga et al., 2019). Minimus data set, TICS, and CNTAB are general scales but used

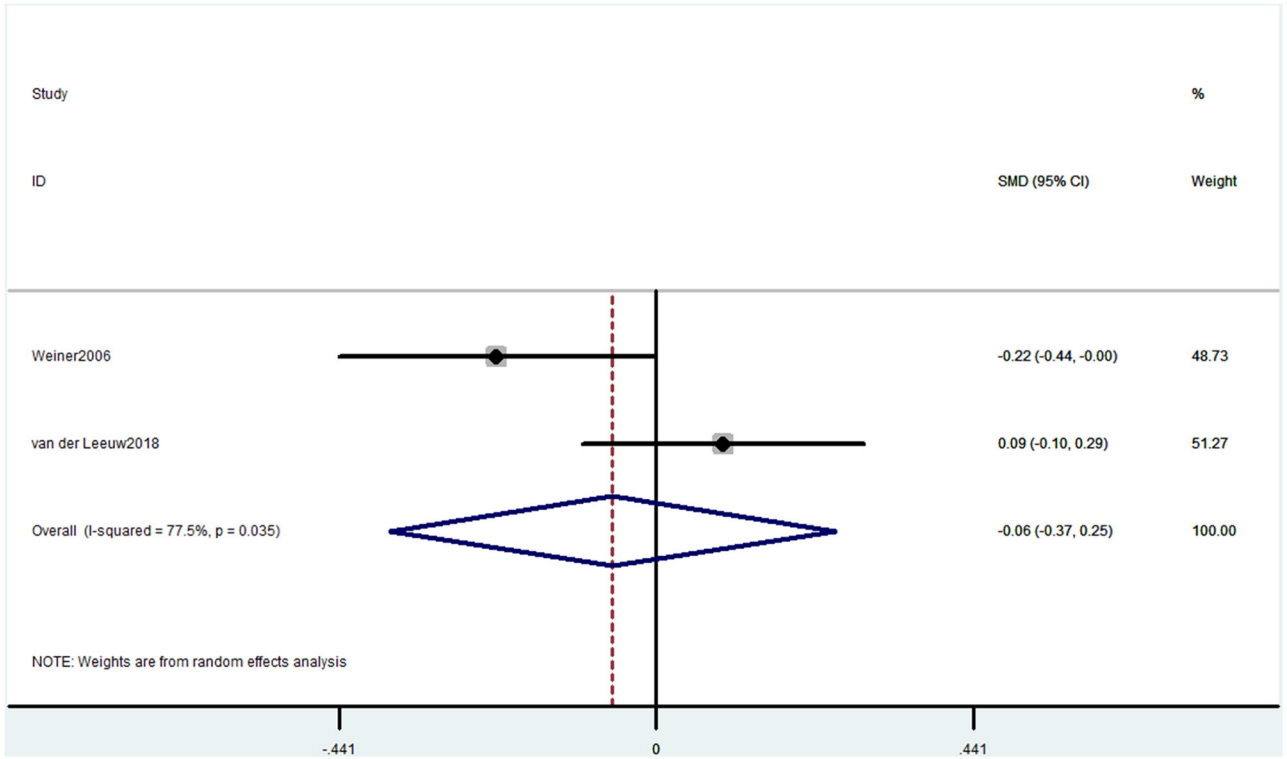

FIGURE 9 | Forest plot of the SMD and 95% CI from a random-effects meta-analysis for the association between chronic pain and incidence of cognitive decline when RBANS was used. The rhombi represent the pooled SMD for this association.

with low frequency (Pickering et al., 2014; Jordan et al., 2018; Van Der Leeuw et al., 2020). Therefore, each of these methods has its own advantages and disadvantages. Hopefully, future studies would compare these methods to confirm their accuracy for investigating.

Our meta-analysis also showed some negative results, possibly due to the cognitive evaluation method adopted. Mini-Mental State Examination was the essential evaluation of cognitive decline. However, only when the cognitive impairment is severe enough, this test works. Also, there have been some studies that suggested MMSE is less accurate than MOCA (Roalf et al., 2013; Pinto et al., 2019). As for RBANS, only two studies were included; the sample size was too small to account for the result. Besides, RBANS is a complex assessment containing many aspects (Weiner et al., 2006), and only one part of the two studies was identical. Also, one recent meta-analysis has found this controversial conclusion between persistent pain and cognitive function among the olds (De Aguiar et al., 2020). However, they did not care about the different cognitive evaluation methods. Therefore, we have a concern about their conclusion. It is important to make it comparable. Our risk of bias assessments has provided some recommendations for future studies. For example, acknowledged diagnostic criteria should be used for CP and cognitive decline. Also, it is critical for the future study to consider the following aspects, such as much more accurate screening of psychiatric disorders, blinded method, and calculation of sample size in advance. In addition, a clinical study of a large sample size should be performed, as many studies included only had small sample sizes.

CONCLUSION

In the present study, we had found that CP might be associated with cognitive decline when the cognitive, evaluative scales were used, including the SF-36 mental component summary, MOCA, PVT, and OSPAN. Future studies are needed with a unified cognitive evaluation method to clarify the association between CP and cognitive function.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

CC and TZ: conception and design. CC: administrative support. XZ, RG, QZ, and CZ: collection and assembly of data. XZ, RG,

and HC: data analysis and interpretation. All authors: manuscript writing and final approval of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.737874/full#supplementary-material

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A Noradrenergic Lesion Attenuates Surgery-Induced Cognitive Impairment in Rats by Suppressing Neuroinflammation

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Postoperative cognitive dysfunction (POCD) is a common postoperative neurocognitive complication in elderly patients. However, the specific pathogenesis is unknown, and it has been demonstrated that neuroinflammation plays a key role in POCD. Recently, increasing evidence has proven that the locus coeruleus noradrenergic (LCNE) system participates in regulating neuroinflammation in some neurodegenerative disorders. We hypothesize that LCNE plays an important role in the neuroinflammation of POCD. In this study, 400 μg of N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) was injected intracerebroventricularly into each rat 7 days before anesthesia/surgery to deplete the locus coeruleus (LC) noradrenaline (NE). We applied a simple laparotomy and brief upper mesenteric artery clamping surgery as the rat POCD model. The open field test, novel objection and novel location (NL) recognition, and Morris water maze (MWM) were performed to assess postoperative cognition. High-performance liquid chromatography (HPLC) was used to measure the level of NE in plasma and brain tissues, and immunofluorescence staining was applied to evaluate the activation of microglia and astrocytes. We also used enzyme-linked immune-sorbent assay (ELISA) to assess the levels of inflammatory cytokines and brain-derived neurotrophic factor (BDNF). Pretreatment with DSP-4 decreased the levels of systemic and central NE, increased the level of interleukin-6 (IL-6) in the plasma at 6 h after the surgery, decreased the concentration of IL-6 in the prefrontal cortex and hippocampus, and decreased the level of interleukin-1β (IL-1β) in the plasma, prefrontal cortex, and hippocampus at 1 week postoperatively. In addition, DSP-4 treatment attenuated hippocampal-dependent learning and memory impairment in rats with POCD, with a downregulation of the activation of microglia and astrocytes in the prefrontal cortex and hippocampus. In conclusion, these findings provide evidence of the effects of LCNE in modulating neuroinflammation in rats with POCD and provide a new perspective in the prevention and treatment of POCD.

Keywords: postoperative dysfunction, locus coeruleus noradrenergic system, neuroinflammation, DSP-4, microglia

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INTRODUCTION

Postoperative cognitive dysfunction (POCD) is one of the most common postoperative complications in elderly patients (Granger and Barnett, 2021) and is characterized by declines in learning, memory, attention, and executive capability following anesthesia and surgery (Chen et al., 2019; Yan et al., 2020). Clinical evidence indicates that POCD may contribute to increased mortality, decreased quality of life, prolonged hospitalization, and increased burden on the medical care system (Steinmetz et al., 2009). However, to date, the neuropathogenesis of POCD remains unknown.

Neuroinflammation has been demonstrated to play a vital role in the occurrence and development of POCD (Soriano et al., 2017; Subramaniyan and Terrando, 2019; Yang et al., 2019). Surgical trauma stimulates the innate immune system, leading to the release of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β), in the systemic circulation (Hirsch et al., 2016), which ultimately compromises the blood–brain barrier (BBB) and promotes monocyte-derived migration of macrophages into the brain parenchyma (Lv et al., 2010), resulting in the activation of microglia and astrocytes (Xu et al., 2017; Hu et al., 2018). Overactivated microglia elicit detrimental effects by the overexpression of cytokines, and reactive astrocytes acquire toxic functions and lose neurotrophic functions (Kaur et al., 2019). The interaction between peripheral and central immune responses aggravates neuroinflammation and neuronal damage, which ultimately impairs cognitive function (Terrando et al., 2011; Alam et al., 2018).

The locus coeruleus (LC) is the main noradrenergic nucleus of the brain and releases the neurotransmitter norepinephrine in many anatomically and functionally diverse brain regions (Schwarz and Luo, 2015; Benarroch, 2018). The locus coeruleus noradrenergic (LCNE) pathway has been proven to be involved in regulating a wide range of advanced cognitive functions (Sara, 2009; Betts et al., 2019), such as working memory, learning and attention (Robbins, 1984; Aston-Jones and Cohen, 2005; Mather et al., 2016), memory formation and consolidation (Gibbs and Summers, 2002; Gibbs et al., 2010; Hansen, 2017), and immunological mechanisms in the brain (Polak et al., 2011; Stowell et al., 2019). A few studies have shown that the LCNE pathway contributes to the pathomechanism of neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD) (Giorgi et al., 2020), and septic encephalopathy (O'Neill et al., 2020), in which neuroinflammation plays an important role. In Termpanit's study, the losses of LC fibers and noradrenaline (NE) exacerbate spatial learning and memory deficits in transgenic mouse models of AD, accompanied by the activation of microglia and astrocytes in the hippocampus (Chalermpalanupap et al., 2018). In primary rat cortical microglial cells, exogenous NE decreased lipopolysaccharide- (LPS-) induced microglial nitric oxide synthase (NOS2) expression and IL-1β production, which was mediated by a β_2 adrenergic receptor (β_2-AR) (Dello Russo et al., 2004).

To date, the effects of the LCNE system on the neuroinflammatory mechanism of POCD have not been reported. Based on the crucial role of LCNE in neuroinflammation-related neurodegenerative diseases, we hypothesize that the LCNE system contributes to the neuroinflammation of rats with POCD.

N-(2-Chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) is a competitive inhibitor of NE uptake and selectively degenerates noradrenergic neurons originating from the LC, whereas noncerulean-innervated noradrenergic axons are unaffected (Fritschy and Grzanna, 1991; Kudo et al., 2011). Thus, DSP-4 has been widely used as a noradrenergic neurotoxin. In this study, we adopted intracerebroventricular administration of DSP-4 at 400 μg per rat (Archer et al., 1984; Chan et al., 1991) to investigate the effects of LCNE lesions on neuroinflammation and cognitive function in POCD model rats.

MATERIALS AND METHODS

Animals and Groups

All procedures were approved by the Animal Ethics Committee of Zhongnan Hospital of Wuhan University, Hubei, China, and all experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The ethical number of Animal Using Protocol (AUP) is WP202008006. Efforts were made to minimize the number of animals used. Wistar rats (12 weeks old, male, weighing 250–350 g) were purchased from SPF (Beijing) Biotechnology Co. Ltd. (Beijing, China). All animals were group-housed four per cage with free access to food and water. The temperature, humidity, and day–night cycle were maintained according to the standards established by the experimental animal laboratory at Zhongnan Hospital of Wuhan University. The rats were allowed 1 week to acclimatize to the laboratory environment before the experiment.

Rats were randomly divided into four groups: a vehicle (V) group (n = 10), a DSP-4 (D) group (n = 10), a vehicle + surgery (V + S) group (n = 18), and a DSP-4 + surgery (D + S) group (n = 18).

Surgery

Rats in Groups V + S and D + S received a simple laparotomy and brief upper mesenteric artery clamping surgery (Hovens et al., 2013, 2014). Specifically, each rat was induced with 3% sevoflurane in 100% oxygen in a transparent acrylic chamber. Fifteen minutes after induction, the rats were removed from the chamber and placed on a heating pad to keep their body temperature between 37 and 38°C. Sevoflurane anesthesia was maintained *via* a cone device, and a 16-gauge needle was inserted into the cone near the nose of the rat to monitor the concentration of sevoflurane. A longitudinal midline incision of approximately 3 cm was made on the skin, abdominal muscles, and peritoneum. Then, the gastrointestinal artery was exteriorized, and the upper mesenteric artery was clamped for 30 min. Clamping the upper mesenteric artery results in a restricted flow to the mesenteric vascular bed although the presence of collateral arteries allows some perfusion

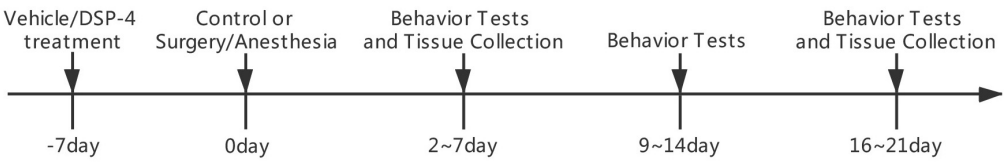

FIGURE 1 | Diagram of the experimental design. Groups V+S and D+S were operated on 7 days after intracerebroventricular injection. Behavioral tests 1, 2, and 3 weeks after the surgery, including a series of tests, started on days 2, 9, or 16 and lasted for 5 days. Rats were sacrificed 1 day after the last behavioral tests in the 1st and 3rd week after the surgery.

(Hovens et al., 2014). Then, the incision was sutured layer-by-layer with 3–0 Vicryl thread, and the rats were put back into an anesthesia chamber for up to 1 h. A heat pad was used to keep their body temperature between 36 and 37°C during the surgery. After recovering from anesthesia, the rats were returned to their home cage with food and water available. The rats in Groups V and D did not receive anesthesia or surgery. All rats with surgery received postsurgical analgesia (flunazine 2.5 mg/kg daily) for 48 h.

Drug Preparation and Injection

N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (neurotoxin DSP-4 hydrochloride, MCE, HY-103210) was given at 400 μg/rat and dissolved in sterile saline 0.9%. Rats were anesthetized with chloral hydrate (300 mg/kg, i.p.). DSP-4 was injected slowly in a volume of 10 μl into the right lateral ventricle (rostral-caudal: −0.8 mm relative to bregma; medial-lateral: −1.8 mm; dorsal-ventral: −3.6 mm from the skull) 7 days before the surgery, and the coordinate was based on The Rat Brain in Stereotaxic Coordinates, the sixth edition of Elsevier Press. The rats in Groups V and V + S were injected with the same volume of sterile saline (0.9%).

Behavioral Tests

Behavioral tests included open field tests, novel objection and novel location (NL) recognition, and Morris water maze (MWM) tests at 1, 2, and 3 weeks postoperatively (**Figure 1**) (Hovens et al., 2014).

Open Field

The open field test was performed to assess motor function and exploratory function. The test was performed 2, 9, and 16 days after anesthesia and surgery. Specifically, the rat was placed in the center of an open field chamber (100 cm × 100 cm × 100 cm) under dim light and was allowed to move freely for 5 min, and the field was divided into a center area (60 cm × 60 cm), four corner areas (20 cm × 20 cm), and four side areas (20 cm × 60 cm). The activities were automatically recorded by a video camera connected to the Any-Maze animal tracking system software (Xinruan Information Technology Co. Ltd., Shanghai, China), and movement parameters were calculated by the software. The total distance moved was recorded and analyzed.

Novel Objection and Novel Location Recognition

The novel objection and novel location recognition test was performed to assess visual and spatial short memory (Wang et al., 2016). The test was performed 3, 10, and 17 days after anesthesia and surgery. Specifically, the day before testing, rats were placed into the testing box (50 cm ×50 cm × 40 cm) two times to acclimate for 5 min. On the testing day, the test consisted of three phases of 3 min, separated by a 45-s pause, and the rat remained in the testing box. In the exploration phase, the rat was presented with two identical objects (plastic bottles filled with water). In the novel object (NO) recognition phase, the rat was presented with a familiar object and a NO. In the novel location (NL) recognition phase, the familiar object from NO did not change its location, while the novel object from NO was placed in a NL. All apparatuses were cleaned with 75% alcohol to remove odors. The time spent exploring objects was recorded by a video camera connected to the Any-Maze animal tracking system software (Xinruan Information Technology Co. Ltd., Shanghai, China), and movement parameters were calculated by the software. The ratio of time the animal spent exploring the novel or relocated object compared to the total object exploration time was considered as a measure of object or location recognition. Trials in which the rats spent less than 5 s exploring the objects were removed from further analysis.

Morris Water Maze

The MWM test was performed to assess spatial learning, spatial memory, and cognitive flexibility. The MWM consists of a circular pool with a diameter of 1.5 m at a depth of 45 cm. The tank was divided into four equal quadrants, with the platform (10 cm × 10 cm) located in the center of the target quadrant. The platform was hidden 1 cm below the water surface, and black ink was used to make the water opaque. The testing room was illuminated with constant light source intensity during the experiments, and different figures and objects that could create visual clues for the rats were hung on the walls. Every rat's swimming track was recorded by a camera above the maze. Each rat was placed randomly in the pool.

The water maze protocol started 4, 11, and 18 days postoperatively, consisting of two training phases, two testing phases, and reversal training over a period of 3 days.

On the 1st day of the protocol, the rats were trained to find the hidden platform. The first training phase included three training sessions with an interval of 1 h, and a training session consisted of three continuous trials. The rat was sequentially placed in each quadrant without a platform and allowed to search for the platform. The latency time was recorded as 60 s if the rat failed to find the platform. Each rat was allowed to stand on the platform, and the surroundings were observed for 10 s. The average escape latency of each training session was considered as a measure to assess spatial learning capacity.

On the 2nd day, the protocol was performed to assess spatial memory capacity, including a testing phase and two training sessions. In the testing phase, the platform was removed, and the rat was placed in a random quadrant and allowed to explore the maze for 60 s. The swimming paths, time spent in each quadrant, and distance moved were recorded by a camera above the maze. Time spent in the target quadrant was considered to measure spatial memory. One hour later, the rats underwent two training sessions to ensure that all the rats learned the location of a platform and had a base as the second testing and reversal training session on the 3rd day. The average escape latency of each training session was considered as a measure to assess spatial learning. We used the average escape latency of five training sessions to draw a learning curve.

The 3rd day of the protocol included the second testing phase and four continuous reversal tests. The second testing phase was the same as the previous day and was performed to assess spatial memory. The reversal tests were performed 1 h later, in which the platform was moved to an opposite quadrant. The average escape latency was taken to measure cognitive flexibility.

Tissue Harvest

The rats were anesthetized and euthanized immediately or 7 or 21 days postoperatively, and the hippocampus and prefrontal cortex were harvested and stored at −80°C for future use. We collected 3 ml of whole blood by cardiac puncture under anesthetic. Blood was centrifuged at 2,500× *g* for 10 min at 4°C, and the plasma was collected at −80°C for further use.

High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) was conducted on plasma and prefrontal cortex and hippocampal tissue homogenates as discussed in previous publications (Finnell et al., 2019) 7 days after the operation. Briefly, all samples were spiked with 1 ng/ml 3,4-dihydroxybenzylamine hydrobromide (DHBA, Sigma, St. Louis, MO, United States, 858781) to serve as an internal standard, mixed with perchloric acid (4 mol/L), and centrifuged at 1,500× *g* for 15 min at 4°C. Supernatant was collected, in which 30 μl of pickled alumina and 1.5 ml of Tris-HCL (1.5 mol/L, pH 8.6, 1% EDTA-Na_2) were added, and the sample was centrifuged at 1,500× *g* for 15 min at 4°C. The supernatant was discarded, the pellet was washed three times with water, the supernatant was collected, and the sample was shocked with 0.1 mol/L perchloric acid and centrifuged at 1,500× *g* for 15 min at 4°C. One hundred microliters of supernatant were used for a HPLC analysis, and all NE data were normalized to DHBA. According to the peak area and standard concentration, the content of norepinephrine in the samples was calculated by the recovery of the internal standard.

Enzyme-Linked Immune-Sorbent Assay

Rat ELISA kits to detect IL-6 (ELK Biotechnology, Wuhan, China, ELK1158), IL-1β (ELK Biotechnology, Wuhan, China, ELK1272), and brain-derived neurotrophic factor (BDNF) (ELK Biotechnology, Wuhan, China, ELK5459) were used to evaluate the concentrations of IL-6, IL-1β, and BDNF in the plasma, prefrontal cortex, and hippocampus.

Immunofluorescence

Seven days after the surgery, each rat was anesthetized with 1.4% isoflurane and perfused transcardially with ice-cold 0.1 M PBS followed by 4% PFA in 0.1 M PBS at pH 7.4. Brains were harvested and fixed in 4% PFA in 0.1 M PBS at 4°C, cryoprotected in 30% sucrose for 72 h, frozen in TissueTek OCT (Sakura), and cut sequentially to 20 μm. After washing in PBS and permeabilization in 0.5% Triton X-100, the sections were blocked with 10% goat serum for 2 h at room temperature to block nonspecific binding and were washed in PBS. Then, the sections were incubated with rabbit anti-Iba-1 (1:200, Abcam, Cambridge, United Kingdom, ab178847) or mouse anti-glial fibrillary acidic protein (GFAP) (1:500, Invitrogen, Waltham, MA, United States, MA5-12023) primary antibodies at 4°C overnight. After washing, the sections were incubated with secondary antibody (goat anti-rabbit) conjugated with CY3 (1:400) or secondary antibody (goat anti-mouse) conjugated with Alexa Fluor dye 488 (1:200) from Invitrogen at room temperature for 2 h in the dark. Immunolabeled sections were coverslipped with 40,6-diamidino-2-phenylindole (DAPI; Invitrogen, Waltham, MA, United States) and analyzed by microscopy (Olympus, Tokyo, Japan) equipped with an imaging system. Five high magnifications were chosen in three nonoverlapping fields randomly acquired in hippocampal and prefrontal cortex subregions using a counting frame size of 0.4 mm^2. Images were processed, and the area of the microglia was quantified using the ImageJ software (NIH). The area of the selected cells was converted into immunoreactivity, which was calculated as the percentage area density defined as the number of pixels (positively stained area) divided by the total number of pixels (the sum of positively and negatively stained area) in the imaged field.

Statistical Analysis

Statistical analysis was performed with SPSS 23.0 (IBM, New York, NY, United States) or GraphPad Prism 6 (GraphPad, New York, NY, United States). Quantitative data are expressed as the means ± SEM. Statistical significance was determined using one-way or two-way ANOVA followed by Tukey's *post hoc* multiple comparison tests. The value of $p < 0.05$ was considered statistically significant.

RESULTS

Effects of ICV Injection of DSP-4 on the Level of Noradrenaline

To confirm the effect of ICV administration of DSP-4, HPLC detection was conducted to measure the levels of NE in the plasma, prefrontal cortex, and hippocampus 7 days after the operation. HPLC results showed that DSP-4 injection decreased NE by 63.15% in the prefrontal cortex (average ± SEM (ng/L): vehicle: 686.75 ± 13.14; DSP-4: 253 ± 24.45), 82.07% in the hippocampus (average ± SEM (ng/L): vehicle:

1,482.25 ± 23.73; DSP-4: 265.75 ± 31.69), and 64.32% in the plasma (average ± SEM (ng/L): vehicle: 171 ± 7.78; DSP-4: 61 ± 3.85) ($p < 0.05$, **Figure 2**). These results confirm that DSP-4 treatment successfully depleted NE in the hippocampus and frontal cortex.

Effects of ICV Injection of DSP-4 on Motor Function and Nonhippocampal-Dependent Memory

There were no significant differences in the distance moved in the open field test and MWM among the four groups ($p > 0.05$, **Figures 3A,B**, **4A,B**, **5A,B**) at 1, 2, and 3 weeks postoperatively, indicating that surgery or DSP-4 treatment does not affect motor function in rats.

No significant differences were found in escape latency during the MWM reversal trials ($p > 0.05$, **Figures 3C**, **4C**, **5C**) or the time spent exploring NOs during the NO phase ($p > 0.05$, **Figures 3D**, **4D**, **5D**), which suggested that nonhippocampal-dependent memory was not influenced by surgery or NE lesions.

Effects of ICV Injection of DSP-4 on Hippocampal-Dependent Learning and Memory

Hippocampal-dependent learning and memory included training sessions in the MWM, time spent in the target quadrant during the testing phase in the MWM, and time spent exploring the relocated objects during the NL phase. To determine the effect of surgery, DSP-4 treatment, and the interactions on cognitive impairment, we used two-way ANOVA and Tukey's *post hoc* multiple comparison tests to analyze behavioral changes.

At 1 and 2 weeks postoperatively, the learning curve (**Figures 3E**, **4E**) of escape latency in the training session showed an obvious downward tendency, indicating that all rats were able to learn where the platform was located. The escape latency in Group V + S was markedly elevated compared with that in Group V, and the pretreatment with DSP-4 diminished the learning impairment caused by surgery. The learning curve showed that training four was the time point with the most obvious difference among the four groups. A two-way ANOVA on the escape latency of training four showed significant effects of the surgery (1 week postoperative: $F_{1,38} = 3.165$, $p = 0.084$; 2 weeks postoperative: $F_{1,32} = 10.974$, $p = 0.002$) as well as the interaction between surgery and DSP-4 treatment (1 week postoperative: $F_{1,38} = 30.333$, $p < 0.001$; 2 weeks postoperative: $F_{1,32} = 7.307$, $p = 0.011$). Subsequent Tukey's *post hoc* analyses indicated that DSP-4 treatment (1 week postoperative: $p = 0.02$; 2 weeks postoperative: $p = 0.001$, **Figures 3E**, **4E**) improves the learning impairment caused by surgery.

According to the result of a two-way ANOVA in time spent in the target quadrant, the interaction of surgery and DSP-4 treatment had a noticeable impact on time spent in target Q at 1 week postoperatively ($F_{1,38} = 14.727$, $p < 0.001$). At 2 weeks after the surgery, surgery ($F_{1,32} = 4.672$, $p = 0.039$) and DSP-4 treatment ($F_{1,32} = 11.181$, $p = 0.002$) both had significant effects. Tukey's *post hoc* analyses showed that compared to Group V, both surgery (1 week postoperative: $p = 0.02$, 2 weeks postoperative: $p = 0.031$, **Figures 3F**, **4F**) and DSP-4 treatment (1 week postoperative: $p = 0.022$, 2 weeks postoperative: $p = 0.007$, **Figures 3F**, **4F**) led to a significantly decreased duration in the target quadrant.

In the NL phase, a two-way ANOVA showed that the interaction of surgery and treatment was the main influencing factor at 1 week postoperatively ($F_{1,38} = 29.982$, $p < 0.001$). Then, we measured Tukey's *post hoc* multiple comparison tests, and the results showed that location recognition was decreased in Groups D and V + S compared to Group V, while the recognition of Group D + S was improved compared to Group V + S (1 week postoperative: $p = 0.032$, 2 weeks postoperative: $p = 0.023$, **Figures 3G**, **4G**).

At 3 weeks postoperatively, there was no significant difference in hippocampal-dependent learning and memory among the four groups ($p > 0.05$, **Figures 5E–G**).

In conclusion, intracerebroventricular injection of DSP-4 attenuated the impairment of hippocampal-dependent learning and memory induced by surgery in rats.

Effects of ICV Injection of DSP-4 on Peripheral and Central Inflammatory Cytokines After Surgery

To assess the effects of DSP-4 on systemic inflammation and neuroinflammation, we measured the concentrations of IL-6

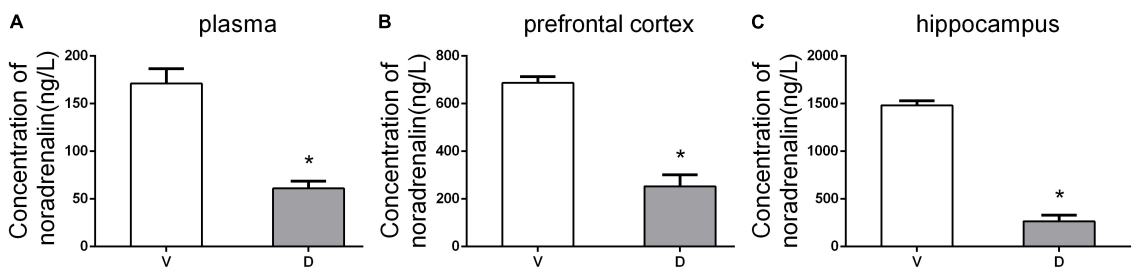

FIGURE 2 | DSP-4 treatment decreased the concentrations of noradrenaline (NE) in the plasma, prefrontal cortex, and hippocampus 7 days postoperatively. **(A)** NE concentrations (ng/L) in plasma. **(B)** NE concentrations (ng/L) in the prefrontal cortex. **(C)** NE concentrations (ng/L) in the hippocampus. The data are presented as the means ± SEM for each group ($n = 5$ per cohort). *$p < 0.05$ compared to Group V.

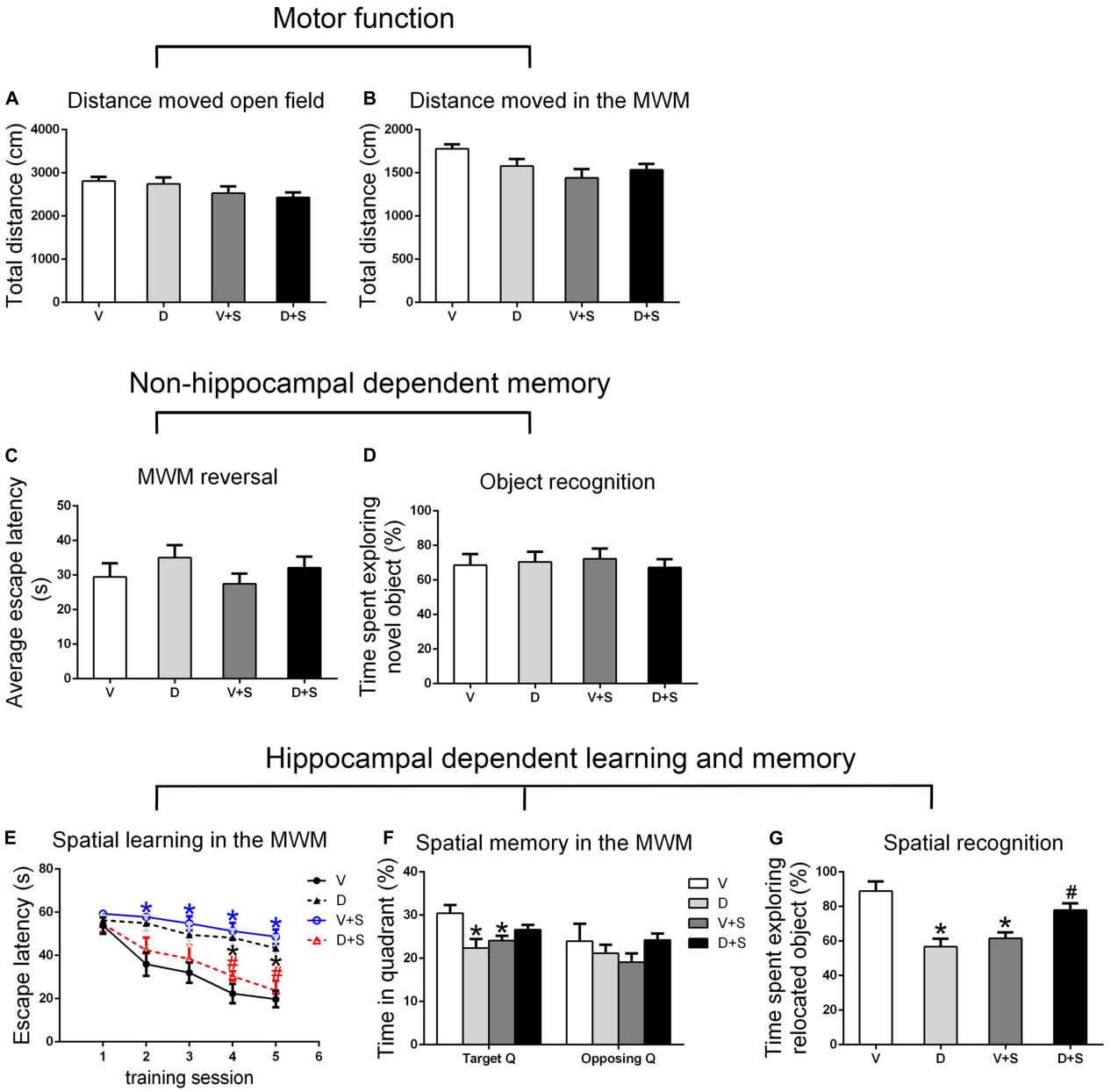

FIGURE 3 | Open field behavior, learning, and memory performance in the four groups 1 week postoperatively. **(A)** Distance moved in the open field test (cm). **(B)** Distance moved (cm) in the Morris water maze (MWM) during the first test as a measure of motor function. **(C)** Cognitive flexibility in the MWM. Average escape latency (s) during the four MWM reversal trials. **(D)** Object recognition in the novel object (NO) test. **(E)** Spatial learning in the MWM. Average escape latency (s) is shown for the five training sessions in the maze. **(F)** Spatial memory in the MWM. The percentages of time in the target quadrant (Target Q) and time in the quadrant opposite to the target quadrant (Opposing Q) are shown. **(G)** Spatial recognition in the novel location (NL) test. Exploration time of the relocated object as a percentage of total object exploration is shown. The data are presented as the means ± SEM for each group (n = 10 per cohort). $^{*}p < 0.05$ compared to Group V, $^{\#}p < 0.05$ compared to Group V + S.

and IL-1β in the plasma, prefrontal cortex, and hippocampus after the surgery. Considering a rapid change in plasma IL-6 (Hovens et al., 2014), we measured plasma IL-6 at 6 h and 1 week postoperatively. At 6 h after the surgery, a two-way ANOVA on the levels of plasma IL-6 showed significant effects of DSP-4 treatment ($F_{1,19}$ = 116.025, $p < 0.001$) and operation ($F_{1,19}$ = 43.923, $p < 0.001$), but there was no significant interaction between treatment and surgery ($F_{1,19}$ = 0.243, $p = 0.629$). Tukey's *post hoc* analysis revealed that both DSP-4 ($p < 0.001$, **Figure 6A**) and surgery ($p = 0.002$, **Figure 6A**) increased the level of IL-6 in plasma at 6 h after the operation. At 1 week postoperatively, there was no significant difference in plasma IL-6 among the four groups ($p > 0.05$, **Figure 6B**).

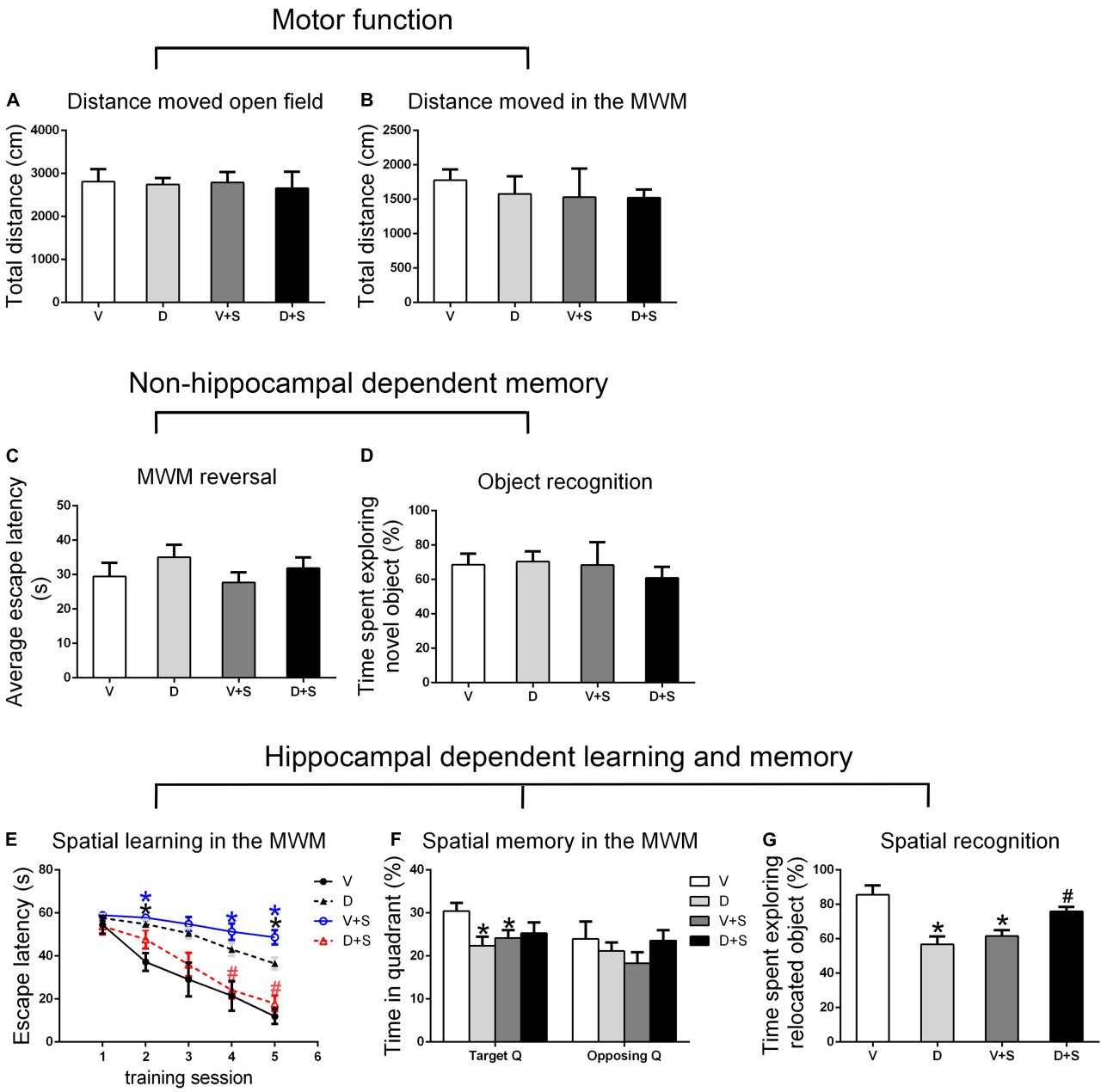

FIGURE 4 | Open field behavior, learning, and memory performance in the four groups at 2 weeks postoperatively. **(A)** Distance moved in the open field test (cm). **(B)** Distance moved (cm) in the MWM during the first test as a measure of motor function. **(C)** Cognitive flexibility in the MWM. Average escape latency (s) during the four MWM reversal trials. **(D)** Object recognition in the NO test. **(E)** Spatial learning in the MWM. Average escape latency (s) is shown for the five training sessions in the maze. **(F)** Spatial memory in the MWM. The percentages of time in the target quadrant (Target Q) and time in a quadrant opposite to the target quadrant (Opposing Q) are shown. **(G)** Spatial recognition in the NL test. Exploration time of the relocated object as a percentage of total object exploration is shown. The data are presented as the means ± SEM for each group (n = 10 per cohort). $^{*}p < 0.05$ compared to Group V, $^{\#}p < 0.05$ compared to Group V + S.

At 7 days after the operation, the results of a two-way ANOVA on IL-6 in the prefrontal cortex showed significant effects of the surgery ($F_{1,15} = 132.18$, $p < 0.001$) and the interaction between treatment and surgery ($F_{1,15} = 218.878$, $p < 0.001$). In the hippocampus, the results of two-way ANOVA showed significant effects of DSP-4 treatment ($F_{1,15} = 31.23$, $p < 0.001$), as well as the interaction between treatment and surgery ($F_{1,15} = 31.23$, $p < 0.001$). Subsequent Tukey's *post hoc* test showed that 7 days after the operation, the levels of IL-6 in Group D (prefrontal cortex: $p = 0.002$, hippocampus: $p = 0.002$, **Figures 6C,D**) were significantly increased compared to those in Group V, and DSP-4 treatment downregulated the elevation of the surgery results (prefrontal cortex: $p < 0.001$, hippocampus: $p = 0.009$, **Figures 6C,D**).

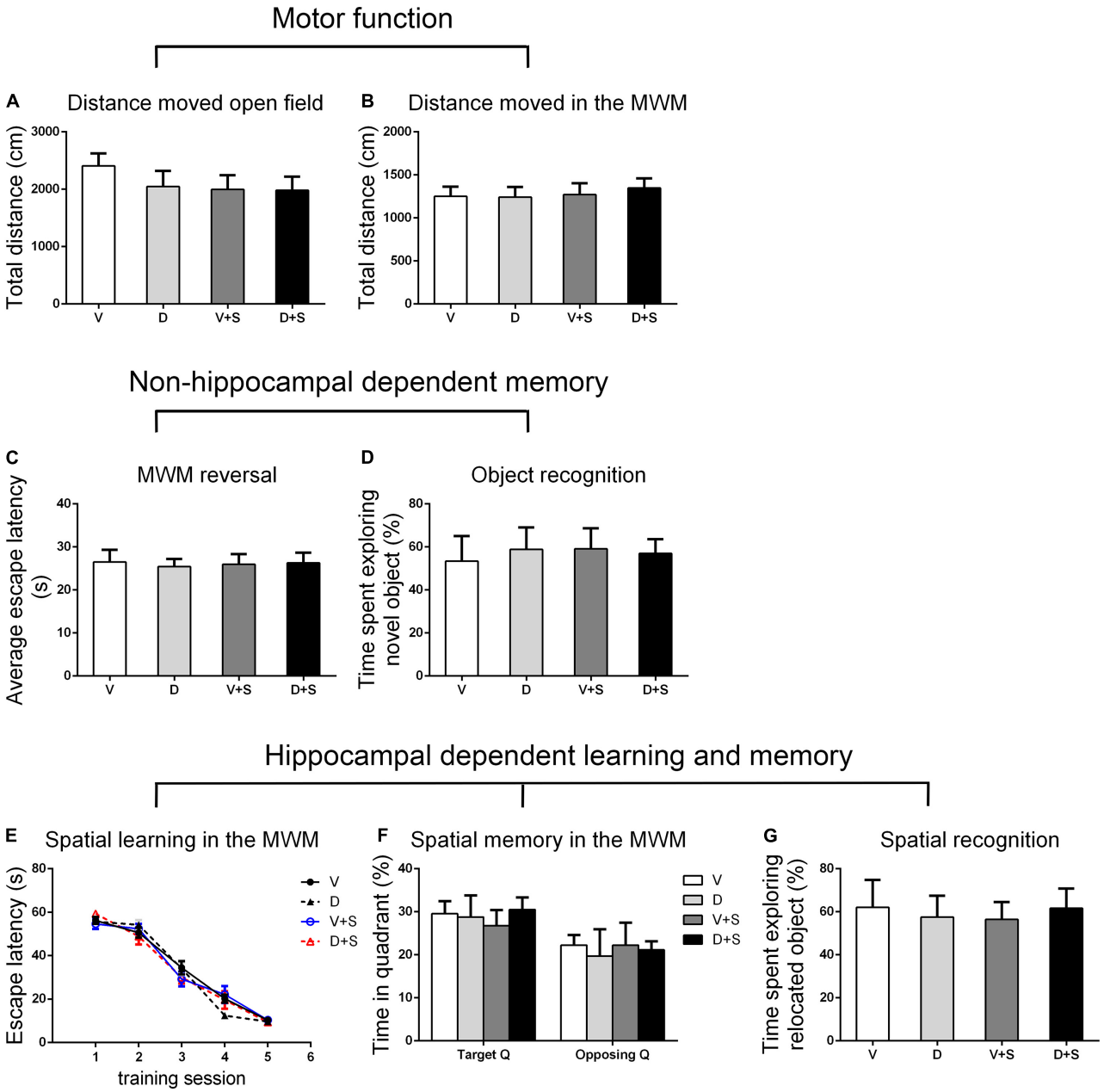

FIGURE 5 | Open field behavior, learning, and memory performance in the four groups at 3 weeks postoperatively. **(A)** Distance moved in the open field test (cm). **(B)** Distance moved (cm) in the MWM during the first test as a measure of motor function. **(C)** Cognitive flexibility in the MWM. Average escape latency (s) during the four MWM reversal trials. **(D)** Object recognition in the NO test. **(E)** Spatial learning in the MWM. Average escape latency (s) is shown for the five training sessions in the maze. **(F)** Spatial memory in the MWM. The percentages of time in the target quadrant (Target Q) and time in the quadrant opposite to the target quadrant (Opposing Q) are shown. **(G)** Spatial recognition in the NL test. Exploration time of the relocated object as a percentage of total object exploration is shown. The data are presented as the means ± SEM for each group (n = 10 per cohort). $^{*}p < 0.05$ compared to Group V, $^{\#}p < 0.05$ compared to Group V + S.

The levels of IL-1β in the plasma, prefrontal cortex, and hippocampus were measured 1 week after the surgery. According to the results of two-way ANOVA, in the plasma, DSP-4 treatment ($F_{1,18}$ = 135.154, $p < 0.001$) and surgery ($F_{1,18}$ = 59.579, $p < 0.001$) affected the level of IL-1β, as well as the interaction between treatment and surgery ($F_{1,18}$ = 435.685, $p < 0.001$). In the prefrontal cortex, the results of two-way ANOVA showed significant effects of the interaction between treatment and surgery ($F_{1,15}$ = 87.278, $p < 0.001$). In the hippocampus, surgery ($F_{1,15}$ = 4.886, $p = 0.047$) and the interaction between treatment and surgery ($F_{1,15}$ = 30.066, $p < 0.001$) affected the level of IL-1β. Tukey's *post hoc* test indicated that surgery significantly increased IL-1β levels in the plasma ($p < 0.001$, **Figure 7A**) and prefrontal cortex

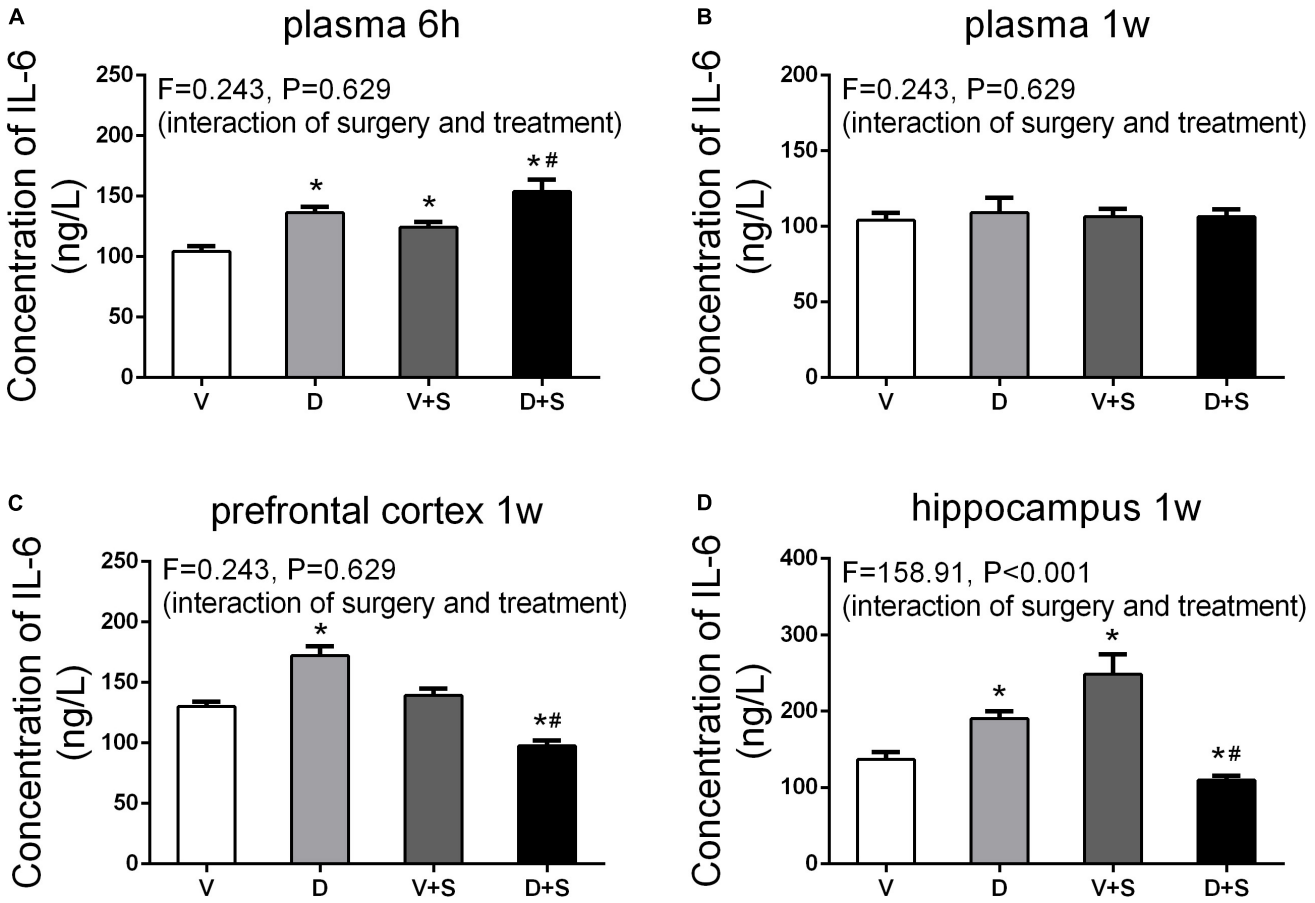

FIGURE 6 | Peripheral and central concentrations of interleukin-6 (IL-6). DSP-4 treatment increased the level of plasma IL-6 under physiological and pathological conditions at 6 h after the surgery **(A)**, while the concentration of IL-6 showed no significant change at 1 week after the surgery **(B)**. At 1 week postoperatively, DSP-4 treatment increased the concentration of IL-6 in the prefrontal cortex **(C)** and hippocampus **(D)** under physiological conditions and decreased it under pathological conditions. The data are presented as the means ± SEM for each group (n = 4 per cohort). $^{*}p < 0.05$ compared to Group V, $^{\#}p < 0.05$ compared to Group V + S.

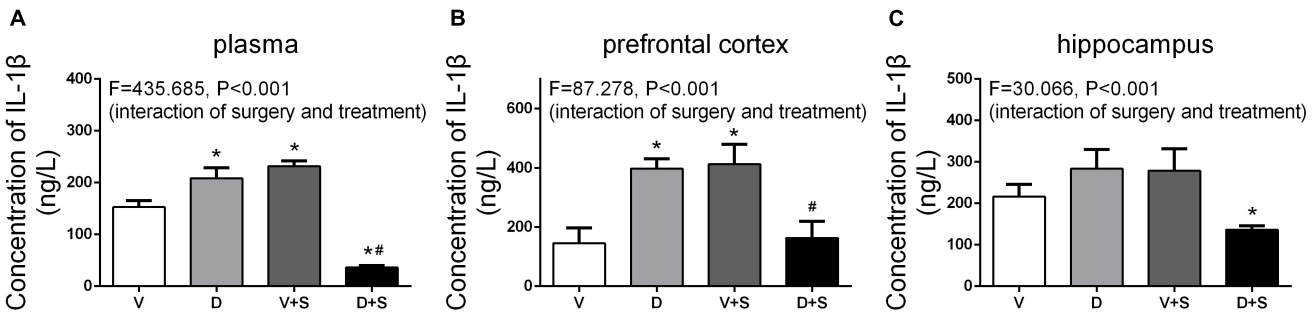

FIGURE 7 | DSP-4 treatment inhibits the increase in interleukin-1β (IL-1β) levels caused by surgery at 1 week postoperatively. Prior to the central administration of DSP-4 attenuated the surgery-induced increase in IL-1β levels in the plasma **(A)**, prefrontal cortex **(B)**, and hippocampus **(C)**. The data are presented as the means ± SEM for each group (n = 4 per cohort). $^{*}p < 0.05$ compared to Group V, $^{\#}p < 0.05$ compared to Group V + S, DP < 0.05 compared to Group D.

($p = 0.006$, **Figure 7B**). In the hippocampus, there was an increasing tendency but no significant difference ($p = 0.455$, **Figure 7C**). Compared to Group V, the IL-1β level of Group D was upregulated in plasma ($p < 0.001$, **Figure 7A**) and the prefrontal cortex ($p = 0.002$, **Figure 7B**), and in the hippocampus, there was an increasing tendency but not a significant difference ($p = 0.295$, **Figure 7C**). The concentration of IL-1β in Group D + S decreased significantly compared to Group V + S in plasma ($p < 0.001$, **Figure 7A**), the prefrontal cortex ($p = 0.009$, **Figure 7B**), and the hippocampus ($p = 0.001$, **Figure 7C**).

Effects of ICV Injection of DSP-4 on the Reactive States of Astrocytes and Microglia in the Hippocampus and Prefrontal Cortex Elicited by Surgery/Anesthesia

We measured the changes in the immunoreactivity of Iba-1 and GFAP in the prefrontal cortex and hippocampus to assess the reactive states of microglia and astrocytes, which represent the major pathological manifestation of neuroinflammation (Norden et al., 2016; Xu et al., 2017; Long et al., 2020).

A two-way ANOVA on Iba-1 densitometry measurements showed significant effects of the surgery (prefrontal cortex: $F_{1,19}$ = 146.337, $p < 0.001$, hippocampus: $F_{1,19}$ = 86.75, $p < 0.001$), as well as the interaction between treatment and surgery (prefrontal cortex: $F_{1,19}$ = 42.089, $p < 0.001$, hippocampus: $F_{1,19}$ = 49.224, $p < 0.001$). Subsequent Tukey's *post hoc* test showed that surgery increased Iba-1 immunoreactivity in the prefrontal cortex ($p < 0.001$, **Figures 8A,C**) and hippocampus ($p < 0.001$, **Figures 8B,D**) compared with Group V, while DSP-4 treatment significantly weakened staining with Iba-1 antibody compared to Group V + S (prefrontal cortex: $p = 0.007$, hippocampus: $p = 0.001$, **Figure 8**). Rats in Group D had increased Iba-1 immunoreactivity in the prefrontal cortex compared to that in Group V ($p = 0.021$, **Figures 8A,C**), but a similar phenomenon was not observed in the hippocampus ($p = 0.066$, **Figures 8B,D**).

The pretreatment with DSP-4 also attenuated the activation of astrocytes. Two-way ANOVA of GFAP densitometry measurements showed significant effects of DSP-4 treatment (prefrontal cortex: $F_{1,19}$ = 17.508, $p = 0.001$, hippocampus: $F_{1,19}$ = 7.520, $p = 0.014$) and surgery (prefrontal cortex: $F_{1,19}$ = 144.457, $p < 0.001$, hippocampus: $F_{1,19}$ = 208.608, $p < 0.001$), as well as the interaction between treatment and surgery (prefrontal cortex: $F_{1,19}$ = 79.111, $p < 0.001$, hippocampus: $F_{1,19}$ = 113.964, $p < 0.001$). Tukey's *post hoc* test revealed that surgery increased GFAP immunoreactivity in the prefrontal cortex ($p < 0.001$, **Figures 9A,C**) and hippocampus ($p < 0.001$, **Figures 9B,D**). DSP-4 treatment decreased the activation of astrocytes in the hippocampus caused by surgery ($p = 0.001$, **Figures 9B,D**); however, in the prefrontal cortex, there was no significant effect on GFAP immunoreactivity between Groups V + S and D + S ($p = 0.126$, **Figures 9A,C**). There was enhanced GFAP immunoreactivity in Group D compared with Group V (prefrontal cortex: $p < 0.001$, hippocampus: $p < 0.001$, **Figure 9**).

Effects of ICV Injection of DSP-4 on Brain-Derived Neurotrophic Factor Levels in the Prefrontal Cortex and Hippocampus

Referencing previous research (Hovens et al., 2014), we selected the time point of 3 weeks postoperatively to measure the concentration of BDNF. The results of two-way ANOVA on BDNF showed significant effects of DSP-4 treatment (prefrontal cortex: $F_{1,15}$ = 62.326, $p < 0.001$, hippocampus: $F_{1,19}$ = 7.188, $p = 0.016$) and surgery (prefrontal cortex: $F_{1,15}$ = 25.013, $p < 0.001$, hippocampus: $F_{1,19}$ = 12.775, $p = 0.003$), as well as the interaction between treatment and surgery (prefrontal cortex: $F_{1,15}$ = 24.860, $p < 0.001$, hippocampus: $F_{1,19}$ = 15.029, $p = 0.001$). Tukey's *post hoc* test showed that both DSP-4 administration (prefrontal cortex: $p < 0.001$, hippocampus: $p = 0.009$, **Figure 10**) and surgery (prefrontal cortex: $p = 0.006$, hippocampus: $p = 0.011$, **Figure 10**) reduced the levels of BDNF in the prefrontal cortex and hippocampus, while DSP-4 treatment had no significant effects on BDNF levels compared to the V + S group (prefrontal cortex: $p = 0.535$, hippocampus: $p = 0.968$, **Figure 10**).

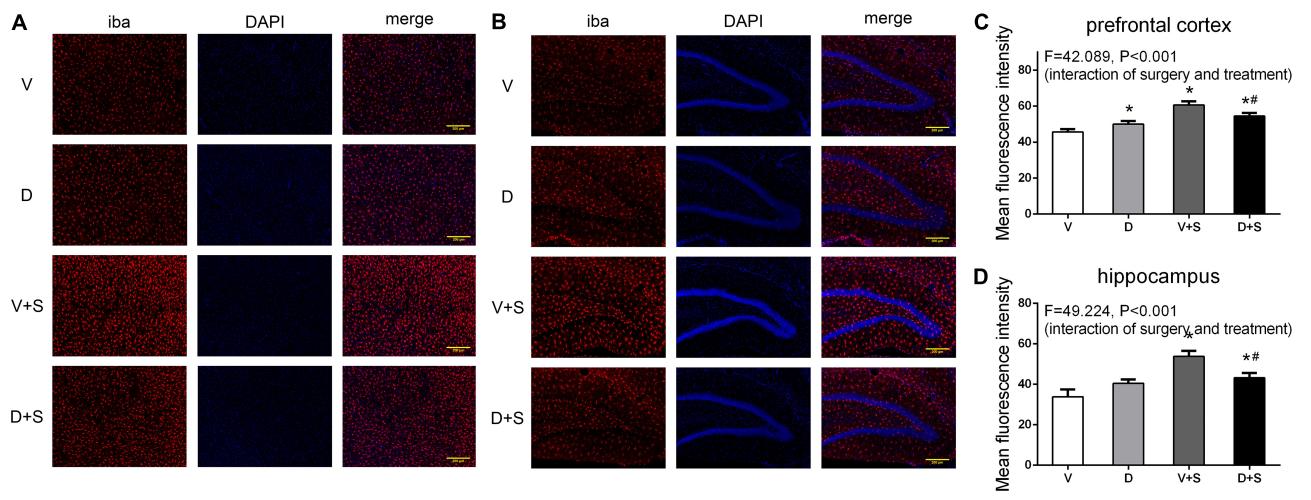

FIGURE 8 | DSP-4 treatment decreased the activation of microglia in surgical rats. The activation of microglia in the prefrontal cortex **(A)** and hippocampus **(B)** at 1 week postoperatively. **(C,D)** Mean fluorescence intensity in the prefrontal cortex and hippocampus at 1 week postoperatively. The data are presented as the means ± SEM for each group (n = 3 per cohort). *Compared to Group V, $^{\#}p < 0.05$ compared to Group V + S.

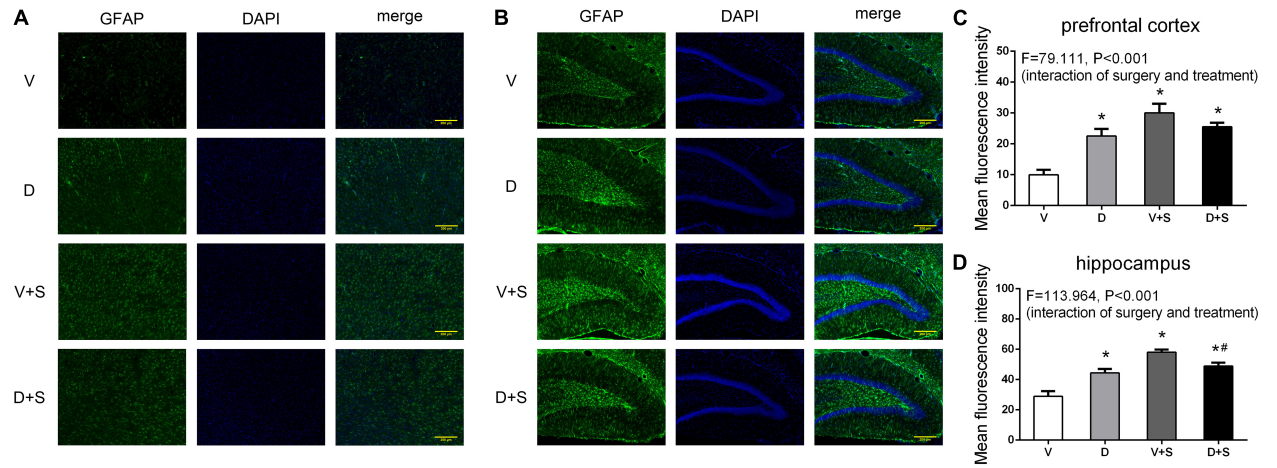

FIGURE 9 | DSP-4 treatment decreased the activation of astrocytes in surgical rats. The activation of astrocytes in the prefrontal cortex **(A)** and hippocampus **(B)** at 1 week postoperatively. **(C,D)** Mean fluorescence intensity in the prefrontal cortex and hippocampus at 1 week postoperatively. The data are presented as the means ± SEM for each group (n = 3 per cohort). $^{*}p < 0.05$ compared to Group V, $^{\#}p < 0.05$ compared to Group V + S, DP < 0.05 compared to Group D.

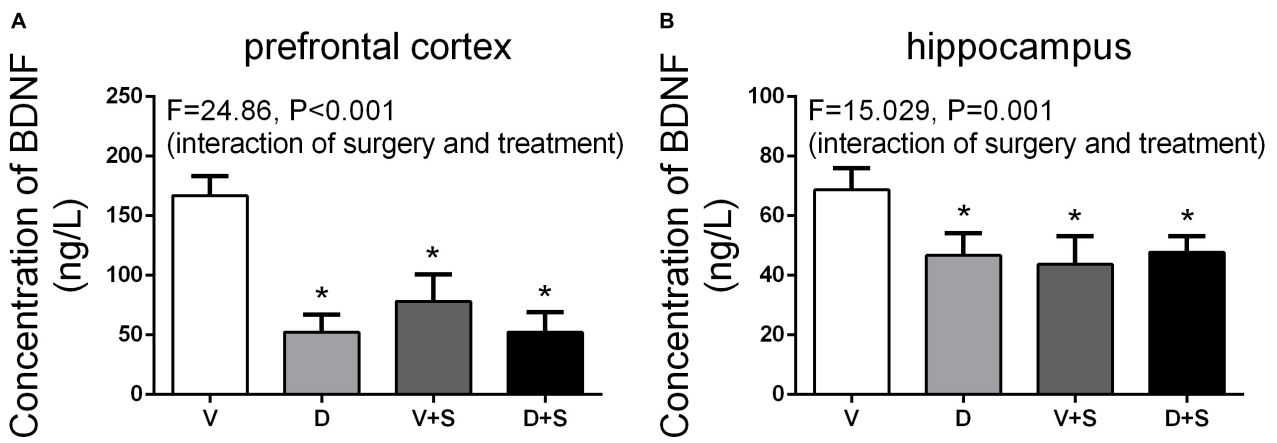

FIGURE 10 | Brain-derived neurotrophic factor (BDNF) levels in the prefrontal cortex **(A)** and hippocampus **(B)** at 3 weeks postoperatively. The data are presented as the means ± SEM for each group (n = 5 per cohort). $^{*}p < 0.05$ compared to Group V, $^{\#}p < 0.05$ compared to Group V + S.

DISCUSSION

The aim of our study was to evaluate the effects of LCNE on surgery-induced neuroinflammation and cognitive decline in rats. We demonstrated that DSP-4, a neurotoxic drug that selectively degenerates noradrenergic neurons originating from the LC, attenuated neuroinflammation in the prefrontal cortex and hippocampus, including decreased levels of proinflammatory cytokines, inhibited the activation of microglia and astrocytes, and improved hippocampal-dependent learning and memory. To our knowledge, this is the first report of the impacts of LCNE on neuroinflammation and cognitive decline in rats with POCD.

A number of studies have shown that neuroinflammation plays a key role in POCD. Peripheral aseptic inflammation is considered to be the beginning of neuroinflammation (Hoogland et al., 2015; Dokalis and Prinz, 2019; Subramaniyan and Terrando, 2019). Sterile surgery leads to the release of damage-associated molecular patterns (DAMPs) followed by the recruitment of leukocytes and the release of inflammatory cytokines (Medzhitov, 2008; McDonald and Kubes, 2011; Zindel and Kubes, 2020). Acute inflammation is necessary to clear the damaged tissue and engage tissue repair; however, an imbalanced immune response can become overwhelming and lead to a "cytokine storm" (Huber-Lang et al., 2018). Overexpressed cytokines cross the BBB, leading to neuroinflammation and a decline in cognition (Terrando et al., 2011).

In the surgery-induced POCD model (Hovens et al., 2014), plasma IL-6 levels were increased significantly the first 12 h after the surgery and decreased to baseline concentrations after 48 h, and the peak concentration occurred at 6 h postoperatively. In addition, the concentration of IL-1β in the prefrontal cortex and hippocampus significantly increased at 1 week postoperatively and returned to a level similar to or even lower than that of the control group at 2 and 3 weeks after the surgery. Our results

demonstrated that in the early stage of inflammation, DSP-4 treatment increased surgery-induced systemic inflammation accompanied by an increased level of plasma IL-6. At 1 week postoperatively, the concentrations of plasma IL-6 returned to baseline; however, the level of plasma IL-1β showed that LCNE lesions had an effect on decreasing the inflammation induced by surgery. DSP-4 pretreatment inhibited systemic inflammation, accompanied by suppressed neuroinflammation, which included decreased levels of IL-6 and IL-1β and the reduced activation of microglia and astrocytes in the prefrontal cortex and hippocampus. Therefore, our research suggests that LCNE lesions mainly play an important anti-inflammatory role in rats with POCD.

These findings are consistent with the proinflammatory roles of LCNE in other disease models associated with neuroinflammation, such as acute and chronic stress (Johnson et al., 2005; Barnard et al., 2019) and Escherichia *coli*-induced septic encephalopathy (Johnson et al., 2008). Pretreatment with DSP-4 blocked the chronic stress-induced elevation in IL-1β in the hippocampus and attenuated the IL-1β increase in circulation (Johnson et al., 2005). In rat peripheral *E. coli* challenge, prior to the central administration of propranolol (a nonselective β receptor blocker) greatly attenuated the *E. coli*-induced increases in IL-1β levels in the brain, tissue, and plasma (Johnson et al., 2008).

These results confirmed that LCNE lesions were beneficial for reducing neuroinflammation, and several studies indicated that the activation of β-AR plays a key role in the effects of NE on neuroinflammation. Sugama et al. (2019) found that acute stress contributes to the activation of microglia in the hypothalamus, hippocampus, and thalamus, and an excitation was substantially inhibited by the β-blocker propranolol but was further activated by pretreatment with the α_2 adrenergic receptor (α_2-AR) blocker yohimbine. In the RAW 264.7 murine macrophage cell line, the activation of β_2-AR leads to IL-1β and IL-6 production through ERK1/2- and p38-dependent activation of ATF-1 and ATF-2 transcription factors, thus playing a proinflammatory role (Tan et al., 2007). However, several studies have indicated that LCNE has an anti-inflammatory role in neuroinflammation. In mice of parkinson's disease, the lesions of LCNE increased the inflammatory activity of microglia and reduced neurotrophic function (Yao et al., 2015). In a study on rat functional pain, continued activation of β-AR resulted in increased levels of TNF-α, IL-6, and IL-1β in CSF and activated microglia and astrocytes in the spinal cord (Zhang et al., 2018). The discrepancy in the effects of LCNE on neuroinflammation may be due to the different animal models and states of the receptors.

The physiological effects of NE are mediated by the three families of G-protein- coupled receptors, α_1, α_2, and β, each consisting of several subtypes. It is worth mentioning that β_2-AR has been proven to be the key to NE regulating inflammation, and NE suppresses TLR-induced pro-inflammatory cytokine TNF-α secretion through β_2-AR (Ağaç et al., 2018). Zhang et al. (2018) found that sustained stimulation of β_2 and β_3 receptors increased the levels of TNF-α, IL-6, and IL-1β in the plasma and CSF, while microglia and astrocytes in the spinal cord were activated. A recent study suggested that α_2-AR also participates in regulating neuroinflammation, and dexmedetomidine treatment prevents LPS-induced cognitive decline and neuroinflammation by inhibiting nuclear factor kappa B (NF-κB) through a pathway mediated by α_2-AR (Li et al., 2020). However, the specific pathophysiological mechanism by which LCNE regulates neuroinflammation is still unclear. Our results showed that under physiological conditions, the depletion of LCNE promoted the release of the central cytokines IL-6 and IL-1β and activated microglia and astrocytes in the prefrontal cortex and hippocampus. In contrast, in pathological settings, LCNE lesions have anti-inflammatory effects. The specific mechanism and reasons remain to be further explored, which may be related to the activation or suppression state of β receptors, and the effects of α_2 receptors should not be overlooked.

The prefrontal cortex in rodents has been demonstrated to participate in advanced functions such as working memory, rule representation, response control, attention, and strategy shifting (Carlen, 2017). Our ELISA and immunofluorescence results suggested that the prefrontal cortex was affected by neuroinflammation with the release of cytokines (IL-6 and IL-1β) and the activation of microglia and astrocytes. However, behavioral outcomes associated with PFC, including NO recognition and MWM reversal tests, showed no significant change. The literature indicates that hippocampus-dependent learning and memory are especially vulnerable to inflammatory insults (Yirmiya and Goshen, 2011; Hovens et al., 2014). This finding may explain why, despite the increased neuroinflammation in the PFC, only hippocampal-dependent functions were impaired in our rats.

Brain-derived neurotrophic factor is involved in plasticity, neuronal survival, the formation of new synapses, and the modulation of excitatory and inhibitory neurotransmitter profiles (Edelmann et al., 2014; Panja and Bramham, 2014; Lima Giacobbo et al., 2019). It has been confirmed that BDNF plays a central role in forms of long-lasting synaptic plasticity associated with the consolidation of hippocampus-dependent memory (Botterill et al., 2015; Patterson, 2015; El Hayek et al., 2019). Referring to a previous study, surgery induced a decreasing concentration of BDNF in the hippocampus 2 weeks after the surgery, and the time point of minimum concentration was 3 weeks postoperatively (Hovens et al., 2014). We measured the levels of BDNF in the prefrontal cortex and hippocampus 3 weeks postoperatively, and the results showed that surgery and DSP-4 treatment downregulated the expression of BDNF when spatial learning and memory returned to baseline levels. Hypothetically, when BDNF was reduced for a long period, a compensatory mechanism was created to protect spatial memory function. Carretón et al. (2012) demonstrated the upregulation of TrkB expression in the hippocampus of $BDNF^{+/-}$ mice, which can partially improve spatial learning and memory performance. This finding suggested that there was a compensatory mechanism to protect cognitive function after prolonged low levels of BDNF.

According to previous research, intraperitoneal injection of 50 mg/kg DSP-4 caused the degeneration of LCNE, which lasted more than 10 weeks (Archer et al., 1984). Chan's results showed that 250 μg of DSP-4 intracerebroventricular injection

was effective in depleting central NE (Chan et al., 1991). DSP-4 is capable of crossing the BBB and does not significantly influence BBB permeability (Tengvar et al., 1989). Our HPLC results suggested that intracerebroventricular injection of DSP-4 at a single dose of 400 μg per rat reduced the release of NE in the plasma, the PFC, and the hippocampus. DSP-4 treatment without surgery damaged hippocampal performance and increased systemic and central inflammation.

There are several limitations to our research. First, we just focused on the effect of LCNE degeneration on regulating systemic and central inflammation, not the receptors or signaling pathways leading to this phenomenon. Further study on modulating the inflammatory mechanism of DSP-4 will give us more ideas for preventing and improving POCD. Second, although DSP-4 is highly selective for LCNE neurons, it can produce minor depleting effects on serotonin (5-HT) (Jonsson et al., 1981). In this experiment, we did not design an indicator to judge the effect of DSP-4 on 5-HT. Nevertheless, the results of previous studies indicated that intraperitoneal injection of DSP-4 at 50 mg/kg does not significantly decrease the level of 5-HT in the CNS (Jonsson et al., 1981; Scullion et al., 2009). Third, we detected Iba-1 densitometry to assess the reactive states of microglia in the prefrontal cortex and hippocampus. Previous research has shown that microglia can produce cytotoxic or neuroprotective effects depending on the phenotypes activated (Tang and Le, 2016; Radandish et al., 2021; Rahimian et al., 2021). Our research evaluated the activation of microglia as a whole but did not distinguish between M1 and M2 phenotypes. Fourth, there might be learning effects due to the repeated behavioral tests. However, on one hand, we believe that the setting up of a vehicle group can counteract the learning effects; on the other hand, after a rest period (generally at least 1 week), by changing the platform location, both learning and relearning experiments can be accomplished (Terry, 2009). In our study, the time interval was 1 week, and the locations of objections in the NO/NL test and a platform in the MWM test were changed at different time points, to reduce possible learning effects. Fifth, POCD is the most common postoperative complication in elderly rats, and we used adult male rats because elderly rats are not readily available. Moreover, the study of Barnard et al. (2019) found that the reactions regulating brain IL-1β using the norepinephrine-β-AR pathway in male and female rats were diverse. Therefore, future investigations should include a comparison of the effects of DSP-4 treatment on neuroinflammation and behavioral changes in rats of different ages and sexes.

In conclusion, our results demonstrated that LCNE lesions increased peripheral inflammation in the early stage of inflammation and decreased neuroinflammation in the middle and advanced stage of inflammation, contributing to the improvement of cognitive function in rats with POCD. Thus, the LCNE system may be a potential therapeutic target for the treatment of POCD, pending further investigation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by The Institutional Animal Care and Use Committee (IACUC) at Practitioner Training institute of Hubei Province.

AUTHOR CONTRIBUTIONS

JW designed and performed the experiment, collected and analyzed the data, and prepared the manuscript. YZ was involved in preparing the animal models and participated in interpreting the results. KL contributed to behavioral testing. XL was involved in biochemical analysis. MG participated in the statistical analysis. MP contributed to the study concept and design, secured funding for the project, and prepared and critically revised the manuscript. All authors reviewed the manuscript.

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Preoperative Chronic Pain as a Risk Factor for Early Postoperative Cognitive Dysfunction in Elderly Patients Undergoing Hip Joint Replacement Surgery: A Prospective Observational Cohort Study

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Background: Although major joint replacement surgery has a high overall success rate, postoperative cognitive dysfunction (POCD) is a common complication after anesthesia and surgery, increasing morbidity and mortality. Identifying POCD risk factors would be helpful to prevent and decrease the occurrence of POCD. We hypothesized that preoperative chronic pain increases the risk of POCD.

Methods: A single-center, observational, prospective cohort study was conducted from January 2018 to March 2020. All consecutive elderly patients (>65 years) who underwent elective total hip arthroplasty or hemiarthroplasty with general anesthesia by the same surgeon were enrolled. The patients underwent neuropsychological testing preoperatively and at 7 days and 2 months after surgery. To determine POCD, a nonsurgical control group was recruited from the general community.

Results: Of the 141 patients who finished the neuropsychological testing 7 days after surgery, 61 (43.2%) had preoperative chronic pain. Of the 61 patients, 17 (27.9%) developed POCD; of the 79 patients with no chronic pain, 10 (12.7%) had developed POCD by 7 days after surgery. Multivariate logistic regression analysis identified preoperative chronic pain as a risk factor of POCD assessed 7 days after surgery (odds ratio 6.527; P = 0.009). There was no significant difference in the POCD incidence 2 months after surgery between patients with and without preoperative chronic pain.

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Conclusion: Preoperative chronic pain was a risk factor of developing POCD within 7 days after surgery in elderly patients following hip joint replacement surgery.

Clinical Trial Registration: [www.ClinicalTrials.gov], identifier [NCT03393676].

Keywords: postoperative cognitive dysfunction, preoperative pain, risk factor, elderly patients, hip joint replacement surgery

INTRODUCTION

Major joint replacement surgery is one of the most common elective procedures and is performed primarily in elderly adults. After major joint replacement surgery, the majority of patients experience substantial relief from both functional disability and pain. Despite the overall success of major joint replacement surgery, patients undergoing this surgical procedure still remain susceptible to cognitive decline, termed POCD, reported rates of which varied between 7% and 75% depending on the definition, assessment tools used, and the population being studied (Deo et al., 2011; Postler et al., 2011). POCD can result in delayed mobilization and discharge from the hospital, worse long-term cognitive dysfunction, and a higher mortality (Rudolph and Marcantonio, 2011).

Several factors have been shown to be risk factors of POCD after hip and knee surgery, including the effects of anesthetics (Duggleby and Lander, 1994; Zywiel et al., 2014), increased age, fewer formal education years or lower reading level, cerebral microemboli caused by fat or marrow entering the blood during surgery (Patel et al., 2010), lower preoperative brain integrity, and lower preoperative executive and memory functions (Monk et al., 2008; Greene et al., 2009; Smith et al., 2009; Price et al., 2014, 2017; Saczynski et al., 2014; Shioiri et al., 2016). However, it is unclear if preoperative pain, which is one of the main reasons of major joint replacement surgery, is also a risk factor of POCD.

The International Association for the Study of Pain defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Treede, 2018). The IASP subcommittee on taxonomy defined it in 1986 as "pain without apparent biological value that has persisted beyond the normal tissue healing time usually taken to be 3 months." Chronic pain was defined as persistent or recurrent pain lasting longer than 3 months (Treede et al., 2015).

It is known that pain directly impairs cognitive function (Price, 2000). Additionally, previous studies have identified the cross-sectional differences in cognition between elderly adults with and without pain (Hart et al., 2000; Moriarty et al., 2011; van der Leeuw et al., 2016). However, no studies have investigated the role of preoperative pain on the occurrence of POCD. In this study, we conducted a single-center, observational, prospective cohort trial in elderly patients who planned to undergo hip joint replacement surgery with general anesthesia to test our hypothesis that preoperative chronic pain is a risk factor of POCD after major joint replacement surgery.

Abbreviations: POCD, postoperative cognitive dysfunction; IASP, The International Association for the Study of Pain; ASA, American Society of Anesthesiologists; MMSE, Mini-Mental State Examination; SD, standard deviation; VAS, visual analog scale; PCIA, patient-controlled intravenous analgesia.

MATERIALS AND METHODS

Study Design

A single-center, prospective, observational cohort study was conducted in Renji Hospital, School of Medicine, Shanghai Jiao Tong University (Shanghai, China), from January 2018 to March 2020. The study was conducted in accordance with the Declaration of Helsinki and was approved by the local ethics committee (RJ198K) (registered at Clinicaltrials.gov: NCT03393676). Written informed consent was obtained from all patients. All investigators were well trained in neuropsychological testing and pain evaluation.

Patient Selection

All consecutive patients who underwent elective total hip arthroplasty or hemiarthroplasty with general anesthesia performed by a single surgeon were enrolled. The inclusion criteria were as follows: (1) >65 years old, (2) speaks Chinese Mandarin, (3) planned to undergo major low limb surgery such as elective total hip arthroplasty or hemiarthroplasty with general anesthesia, (4) signed the informed consent form, and (5) assessed as ASA classifications I to II.

The exclusion criteria included the following: (1) existing cerebral disease or a history of neurological and psychiatric disease, including psychosis, stroke, epilepsy, and Alzheimer's disease; (2) existing cognitive impairment as evidenced by the MMSE scores less than 24; (3) severe hearing or visual impairment; (4) unwillingness to comply with the protocol or procedures; (5) inability to communicate in Chinese Mandarin; (6) presence of serious pulmonary, heart, liver, or renal insufficiency; and (7) had undergone anesthesia or surgery within the past 30 days.

To determine POCD and cognitive decline, it is necessary to use a nonsurgical control group (Lewis et al., 2004). The selection criteria of the subject controls were the same, wherein subjects were matched to the elective total hip arthroplasty or hemiarthroplasty replacement surgery sample by age, gender, and education, but they had no chronic pain. The controls were recruited from the general community. The community control group was recruited over the same time frame and underwent neuropsychological testing at the same time intervals, corresponding to assessments in the patients undergoing surgery.

All patients underwent elective total hip arthroplasty or hemiarthroplasty. All clinical care followed routine clinical practice. All surgical plans were decided and performed in a standard manner by the same orthopedic surgeon.

All patients received general anesthesia according to routine clinical practice. Anesthesia was induced using intravenous (IV) midazolam (0.02 mg/kg), propofol (1–2 mg/kg), sufentanil (0.2–0.5 μg/kg), and rocuronium (0.6 mg/kg). Anesthesia was maintained with inhaled sevoflurane (1MAC) and intravenous propofol (2–3 mg/kg/h), remifentanyl (0.2–0.5 μg/kg/min), and rocuronium (10 μg/kg/min). During anesthesia, radial artery was cannulated for intra-arterial pressure monitoring. Electrocardiograph (ECG), $PETCO_2$, and end-tidal sevoflurane concentration were monitored during operation. Ephedrine was given if mean arterial blood pressure decreased to less than 75% of baseline. Systolic blood pressure was kept between 80 and 90 mmHg.

All patients received standardized perioperative care, including preoperative and intraoperative care and postoperative pain control. We recorded whether patients used nonsteroidal anti-inflammatory drugs preoperatively or not (**Table 1**). However, the dose, duration, and frequency of use of nonsteroidal anti-inflammatory drugs were not assessed in our dataset. All patients received nonsteroidal anti-inflammatory drugs 10 min before the end of surgery. Postoperative PCIA was provided by means of a continuous intravenous infusion of sufentanyl (1.5 μg/kg) plus nonsteroidal anti-inflammatory, which was diluted into 100 ml with normal saline. The pump was programmed as a background infusion of 1 ml/h, a PCIA rescue dose of 2 ml, and a lockout period of 15 min. Tramadol was given on request as rescue analgesia postoperatively when patients reported severe pain. This analgesic schedule include opioid medicine that was just maintained for 48 h.

Visual Analog Scale

The visual analog scale anchored at 0 and 10 was used to measure pain intensity. Scores of 1–3 were designated as grade 2, scores of 4–7 were designated as grade 3, and scores of 8–10 were designated as grade 4. Patients with a baseline pain of at least 4 on a VAS and lasting for more than 3 months were assigned into the chronic pain group, and the patients without chronic pain were assigned to the non-chronic pain group.

POCD Identification

We carried out neuropsychological testing, including a battery of six neuropsychological tests at baseline (the day before surgery) and at 7 days and 2 months after surgery. The neuropsychological tests consisted of the MMSE, Visual Reproduction Test, Digit Span Test (forward, backward), Digit Symbol Test, Color Trail Tests (1 and 2), and Stroop Color and Word Test (A, B, and C). All tests were conducted in the same order at each time point. These measures not only are highly sensitive to the types of cognitive impairments but also have no cultural bias.

The MMSE was only used to exclude patients with existing cognitive impairment (MMSE scores < 24) at baseline. The remaining neuropsychological battery of tests addressed three cognitive domains: attentional capacity, executive function, and memory. In the neuropsychological tests including the Visual Reproduction Test, Digit Span Test (forward and backward), Digit Symbol Test, and Stroop Color and Word Test (A, B, and C), the higher scores reflected better performance. The Color Trail Tests (1 and 2) provided estimates of attentional capacity and executive function, where a shorter time reflected better performance.

Since these tests are prone to the test–retest practice effect, an age-matched control group was tested at the same intervals, providing an indication of practice effect with this test battery for the given time intervals between sessions.

Postoperative cognitive dysfunction was defined as a score decrease of 1.96 SDs below baseline in two or more neuropsychological tests or by a decrease of 1.96 SDs in the combined *Z*-score. The *Z*-score was calculated according to the methods of Rasmussen (Lewis et al., 2004). The formula was as follows:

$$Z = \frac{\triangle X - \triangle X_{control}}{SD(\triangle X)_{control}}$$

$\triangle X$ was obtained from the postoperative score (7 days or 2 months) to the subtracted preoperative score. $\triangle X_{control}$ was calculated from the community controls. $SD(\triangle X)_{control}$ was SD for the changes in test results in the community control group. A combined *Z*-score was calculated as the sum of *Z*-scores divided by the SD for this sum of *Z*-scores in the community control group. Data from the healthy control group were used to gain information on the practice effect and normal distribution in the test results for this age group, and the *Z*-scores were calculated (Rasmussen et al., 2001).

Statistical Analysis

Continuous data are reported in the tables as mean and SD or as median and interquartile range, and categorical data were reported as frequency and percentage. We examined the demographic and health characteristics per dichotomous pain variable ("chronic pain" vs. "non-chronic pain"). Between-group differences were analyzed by using the analysis of variance or the Mann–Whitney rank sum test for continuous measures and the chi-squared test or Fisher's exact test for categorical measures. For univariate analysis, we used the Mann–Whitney *U* test for nonnormal distributions or the independent samples *t*-test for normally distributed continuous variables and the chi-square test for categorical variables to compare differences between the group with postoperative POCD and that without postoperative POCD (7 days and 2 months after surgery). All clinically relevant and statistically significant preoperative variables were then entered into a multivariate logistic regression analysis using a forward entry method to identify independent preoperative risk factors for POCD. The Hosmer–Lemeshow goodness of fit test was performed to evaluate model fitting of the logistic multivariable model. Data are presented as the odds ratio and 95% confidence intervals (CIs). Values of $P < 0.05$ were considered to be indicative of statistical significance.

Sample Size Calculation

Pass (Version 15.0, NCSS, LLC, Kaysville, UT, United States) software was used for the sample size calculation. Logistic

TABLE 1 | General characteristics of patients.

Characteristic	Total, *n* = 141	Non-chronic pain, *n* = 80 (56.7%)	Chronic pain, *n* = 61 (43.2%)	*P*-value
Age (year)	69.6 (6.2)	69.1 (6.2)	70.1 (6.1)	0.347
Gender (male/female)	60/81	34/46	26/35	1.000
BMI (kg/m^2)	24.3 (3.7)	23.1 (3.4)	25.9 (3.4)	<0.001
Education Level (year)	9.0 (9.0–12.0)	9.0 (8.0–12.0)	9.0 (6.0–12.0)	0.079
History of hypertension	68 (48.2)	34 (42.5)	34 (55.7)	0.129
History of diabetes	28 (20.6)	16 (20.0)	12 (19.7)	1.000
History of smoking	14 (9.9)	9 (11.2)	5 (8.2)	0.586
ASA grade I/II	57/84	35/45	22/39	0.390
Diagnosis				<0.001
Osteoarthritis	48 (34.0)	2 (2.5)	46 (75.4)	
Femoral neck fracture	84 (59.6)	77 (96.2)	7 (11.5)	
Aseptic necrosis of femoral head	9 (6.4)	1 (1.2)	8 (13.1)	
Surgery type				<0.001
Total hip arthroplasty	86 (61.0)	37 (46.2)	49 (80.3)	
Hemiarthroplasty	55 (49.0)	43 (53.8)	12 (19.7)	
Surgery time (min)	120.0 (90.0–150.0)	111.3 (89.1–150.0)	150.0 (99.6–180.0)	<0.001
Blood loss (ml)	400.0 (200.0–600.0)	400.0 (200.0–600.0)	400.0 (300.0–600.0)	0.312
Blood transfusion (ml)	400.0 (0.0–600.0)	400.0 (0.0–600.0)	400.0 (0.0–700.0)	0.282
Intraoperative hypotension	98 (69.5)	53 (66.3)	45 (73.8)	0.362
Intraoperative ephedrine use	15.2 (13.9)	13.6 (13.3)	17.2 (14.60	0.138
Length of hospital stay (days)	7.0 (7.0–11.0)	7.0 (7.0–9.0)	8.0 (7.0–13.0)	0.014
Chronic pain period (year)	0.0 (0.0–20.0)	0.0 (0.0)	4.3 (5.0)	<0.001
Preoperative nonsteroidal anti-inflammatory drug use	62 (44.0)	32 (40.0)	30 (49.2)	0.307
Postoperative tramadol use	54 (38.3)	29 (36.3)	25 (41.0)	0.603
Postoperative nonsteroidal anti-inflammatory drugs use (7 days)	91 (64.5)	49 (61.3)	42 (68.9)	0.379
Postoperative nonsteroidal anti-inflammatory drugs use (2 months)	19 (14.2)	9 (12.2)	10 (16.7)	0.468
PCIA	107 (75.9)	60 (75.0)	47 (77.0)	0.844
POCD 7 days	27 (19.1)	10 (12.5)	17 (27.9)	0.030
POCD 2 months	10 (7.5)	6 (8.1)	4 (6.7)	1.000

Values are mean (SD) or median (IQR) or N (%).
POCD, postoperative cognitive dysfunction; PCIA, patient controlled intravenous analgesia.

regression tests for odds ratios with one binary X procedure were performed. With $\alpha = 0.05$, a power of 80%, and an odds ratio = 3.0, the POCD incidence of major joint replacement was approximately 20%, and chronic pain prevalence in such patients was at nearly 50% (Wylde et al., 2015; van der Leeuw et al., 2016). We then estimated that a total of 135 patients would be required for the study. To set the attrition rate at 10%, a total of 148 patients were required.

RESULTS

A total of 155 consecutive patients were enrolled in this study. The trial profile is shown in **Figure 1A**. Patients were excluded for the following reasons: three refused to sign the informed consent form, four baseline MMSE scores were <24, three declined to participate in a later test, two were admitted to an intensive care unit after surgery, and two underwent reoperation in the follow-up period. The remaining 141 (57.4% female) patients were included in the diagnosis of early POCD at day 7. Additionally, two patients failed to show up for their final evaluation at 2 months, two underwent further surgery prior to follow-up, and three had lost data. Hence, the remaining 134 patients' cognitive test results were included at 2 months.

Among the 104 age- and education-matched community adults enrolled for the POCD calculations in this study, nine refused to sign the informed consent, three did not complete the baseline neuropsychological testing, three underwent operations after baseline cognitive evaluation, five declined to continue, and two had lost data. Finally, 82 subjects were included in the statistical analysis (**Figure 1B**).

Patients' General Characteristics

Table 1 presents the patients' general characteristics. At day 7 postoperatively, a total of 27 patients developed cognitive decline, of which 17 had chronic pain preoperatively (27.9%, $P < 0.05$). The BMI of patients with chronic pain was significantly higher than that of those without chronic pain ($P < 0.05$).

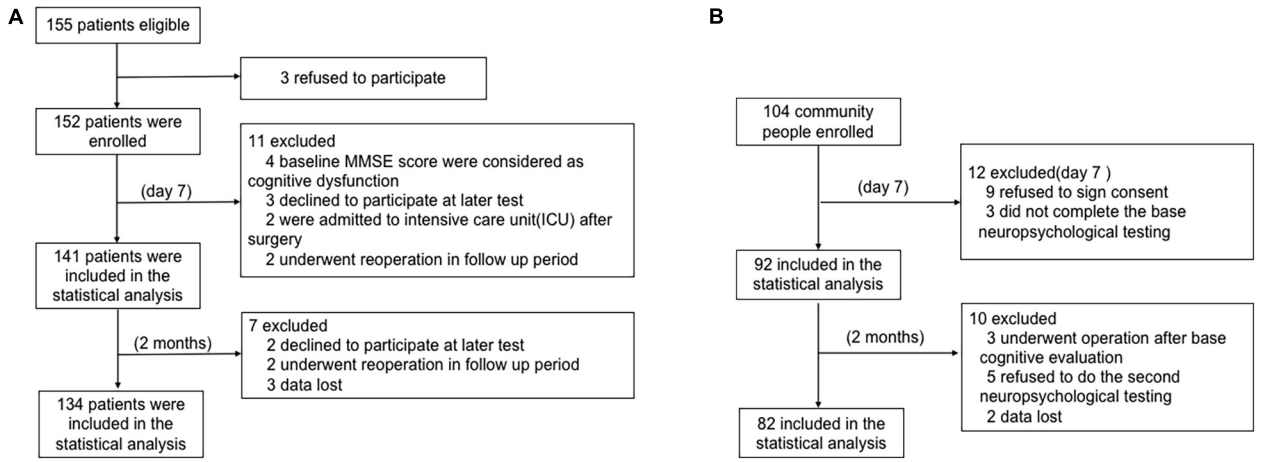

FIGURE 1 | The flow chart of this study. **(A)** Patient flow chart. **(B)** Health community people flow chart.

TABLE 2 | General characteristics of the community people not receiving surgery.

Characteristic	*N* = 82
Age (year)	67.5 (65–82)
Gender (male/female)	29/53
Education level (year)	9.5 (2.7)
History of hypertension	40 (48.8)
History of diabetes	17 (20.7)
History of smoking	6 (7.3)
ASA grade I/II	32/50

Values are mean (SD) or median (IQR) or N (%).

TABLE 3 | VAS scores of the patients.

Time	VAS grade	Non-chronic pain, *n* = 80 (56.7%)	Chronic pain, *n* = 61 (43.2%)	*P*-value
Preoperative	0–2	41 (51.2)	0 (0)	<0.001
	3–4	39 (48.8)	61 (100)	
Postoperative				
3 days	0–2	51 (63.7)	35 (57.4)	0.477
	3–4	29 (36.3)	26 (42.6)	
7 days	0–2	79 (98.8)	56 (91.8)	0.044
	3–4	1 (1.3)	5 (8.2)	
2 months	0–2	69 (93.2)	52 (86.7)	0.284
	3–4	5 (6.8)	8 (13.3)	

Values are N (%).
VAS, visual analog scale.

There was a significant difference in the surgery type, surgery time, and length of hospital stay between patients with chronic pain and patients without chronic pain ($P < 0.05$) (**Table 1**). No significant difference in PCIA use (77.0 vs. 75.0%) and nonsteroidal anti-inflammatory drugs use before (49.2 vs. 40.0%) or after surgery (7 days and 2 months) between patients with chronic pain and patients without chronic pain was found (**Table 1**). **Table 2** presents the general characteristics of the community controls.

VAS Scores of Patients Before and After Surgery

The VAS scores were significantly higher in patients with chronic pain than in those without chronic pain ($P < 0.05$) before surgery and 7 days after surgery. Patients had similar VAS scores after surgery (3 days and 2 months), regardless of whether they had chronic pain or not (**Table 3**). There was no significant difference in analgesic use before surgery and after surgery (3 days, 7 days, and 2 months) between patients with chronic pain and those without chronic pain (**Table 1**). However, VAS score and analgesic use (3 days and 2 months after surgery) of patients with POCD were significantly higher than those of patients without POCD (**Tables 4**, **5**).

Neuropsychological Test Scores of Patients With Chronic Pain and Patients Without Chronic Pain Before and After Surgery

There was significantly ($P < 0.05$) higher scores on the Visual Reproduction Test in patients without chronic pain than in patients with chronic pain before surgery (**Table 6**). Seven days after surgery, scores on the Visual Reproduction Test and Stroop Color and Word Tests B and C were significantly ($P < 0.05$) higher in patients without chronic pain than in patients with chronic pain (**Table 6**). Scores on the Stroop Color and Word Test B were significantly ($P < 0.05$) higher in patients without chronic pain than in patients with chronic pain 2 months after surgery (**Table 6**).

Patients With Chronic Pain Were More Likely to Develop POCD

In total, the incidence of early POCD (7 days postoperative) was 27 (19.1%) per 141 patients. The incidence of late POCD

TABLE 4 | Logistic analysis for the POCD occurred at 7 days after surgery.

Characteristic	Univariate analysis	Multivariate analysis	
	p-value	Odds ratio (95% CI)	*P*-value
Preoperative chronic pain	0.030	6.527 (1.583–26.908)	0.009
Postoperative VAS score (3 days)	0.042	1.726 (0.540–5.519)	0.358
Postoperative tramadol use	0.049	1.561 (0.349–6.983)	0.560
Intraoperative hypotension	0.003	6.608 (0.978–44.660)	0.053
Intraoperative ephedrine use	0.001	1.004 (0.961–1.049)	0.850
Pain period	0.136	0.948 (0.832–1.080)	0.424
BMI	0.509	0.862 (0.739–1.006)	0.059
Surgery time	0.977	1.001 (0.993–1.010)	0.749
Diagnosis	0.193	1.311 (0.451–3.813)	0.619
Surgery type	1.000	0.566 (0.188–1.707)	0.312
Length of hospital stay (days)	0.749	1.050 (0.885–1.246)	0.574

VAS, visual analog scale.

(2 months postoperative) was 10 (7.5%) per 134 patients. Among the 141 patients, 61 (43.2%) had chronic pain preoperatively. Of the 61 patients, 17 (27.9%) developed POCD; of the 79 patients without chronic pain, 10 (12.5%) developed POCD at 7 days after surgery (**Figure 2**).

Among the 134 patients who finished the neuropsychological testing at 2 months after surgery, 60 (44.8%) had chronic pain preoperatively. Of the 60 patients, 4 (6.7%) developed POCD; of the 74 patients without chronic pain, 6 (8.1%) developed POCD at 2 months after surgery.

Chronic Pain Preoperatively Was a Risk Factor of POCD Within 7 Days After Surgery

In the univariate logistic analysis, we found that preoperative chronic pain (P = 0.030), VAS score at 3 days after surgery (P = 0.042), postoperative tramadol use (P = 0.049), the rate of intraoperative hypotension (P = 0.003), and intraoperative ephedrine use (P = 0.001) were risk factors of POCD within 7 days after surgery. For multivariable analysis, all the covariates with $P \leq 0.10$ in the univariate analysis were entered into a backward stepwise logistic regression model for prediction of the primary outcome: incidence of POCD. BMI, diagnosis, surgery type, surgery time, and length of hospital stay were forced into the multivariable model. These variables were selected to account for possible confounding, as there was significant difference in the BMI, diagnosis, surgery type, surgery time, and length of hospital stay between patients with chronic pain and patients without chronic pain ($P < 0.05$) (**Table 1**). However, in the multivariate logistic analysis, only preoperative chronic pain was a risk factor of POCD within 7 days after surgery (odds ratio 6.527, 95% CI 1.583–26.908, P = 0.009; **Table 4**). There was no difference between the patients who had POCD and those who did not in gender, education, ASA grade, surgery time, blood loss, blood transfusion, length of hospital stay, and VAS scores before or after surgery (day 7).

In the univariate logistic analysis, we found that the VAS score at 2 months after surgery (P = 0.037), the rate of intraoperative hypotension (P = 0.034), intraoperative ephedrine use (P = 0.004), and nonsteroidal anti-inflammatory drug use 2 months postoperation (p = 0.035) were risk factors of POCD within 2 months after surgery. In the multivariate logistic analyses, no risk factor for POCD was found within 2 months after surgery (**Table 5**).

DISCUSSION

Our study demonstrated that preoperative chronic pain was a potent risk factor (odds ratio 6.527) for POCD within 7 days postoperatively in elderly patients who had undergone major joint replacement surgery but not for POCD at 2 months after surgery. Previous study showed that chronic preoperative pain impaired recovery of attention after surgery in non-elderly patients (Gu et al., 2019). To the best of our knowledge, the

TABLE 5 | Logistic analysis for the POCD occurred at 2 months after surgery.

Characteristic	Univariate analysis	Multivariate analysis	
	p-value	Odds ratio (95% CI)	*P*-value
Postoperative VAS score (2 months)	0.037	0.514 (0.139 – 1.905)	0.319
Intraoperative hypotension	0.034	0.000	0.998
Intraoperative ephedrine use	0.004	1.047 (0.982 – 1.116)	0.164
Postoperative nonsteroidal anti-inflammatory drug use (2 months)	0.035	29.844 (0.813 – 1095.101)	0.065
Preoperative chronic pain	1.000	1.387 (0.152 – 12.681)	0.772
Pain period	0.567	0.792 (0.509 – 1.232)	0.301
BMI	0.681	1.139 (0.929 – 1.397)	0.211
Surgery time	0.203	0.982 (0.962 – 1.003)	0.099
Diagnosis	0.588	1.126 (0.136 – 9.351)	0.912
Surgery type	1.000	1.870 (0.315 – 11.088)	0.491
Length of hospital stay (days)	0.153	0.992 (0.697 – 1.413)	0.966

POCD, postoperative cognitive dysfunction; VAS, visual analog scale.

TABLE 6 | Neuropsychological test scores of patients before and after surgery.

Neuropsychological test scores Mean (SD)	Community people, *n* = 82	Non-chronic pain, *n* = 80 (56.7%)	Chronic pain, *n* = 61 (43.2%)	*P*-value
Preoperative				
MMSE	27.6 (1.2)	27.5 (1.6)	27.3 (1.8)	0.541
Digit Span Test	12.4 (2.2)	11.4 (2.2)	11.1 (1.8)	0.339
Digit Symbol Test	32.2 (10.8)	28.9 (11.1)	31.9 (12.7)	0.263
Visual Reproduction Test	8.3 (2.7)	7.8 (2.7)	6.8 (2.9)	**0.045**
Stroop Color and Word Test A	80.7 (15.6)	74.2 (16.9)	70.5 (13.9)	0.169
Stroop Color and Word Test B	64.0 (14.7)	55.4 (14.9)	51.2 (15.2)	0.109
Stroop Color and Word Test C	31.9 (10.0)	28.5 (9.2)	27.3 (8.3)	0.406
Color Trail Tests 1	74.8 (33.9)	81.9 (39.1)	87.6 (31.7)	0.361
Color Trail Tests 2	141.1 (63.1)	148.2 (60.4)	160.9 (49.5)	0.186
Postoperative (7 days)				
Digit Span Test	12.8 (2.1)	11.4 (2.2)	10.9 (2.0)	0.119
Digit Symbol Test	36.1 (11.7)	30.4 (12.2)	28.3 (10.3)	0.118
Visual Reproduction Test	8.9 (2.6)	9.0 (2.7)	7.6 (3.0)	0.004
Stroop Color and Word Test A	80.0 (17.0)	71.8 (17.5)	67.8 (14.0)	0.145
Stroop Color and Word Test B	65.3 (14.0)	57.2 (15.2)	52.0 (13.3)	0.034
Stroop Color and Word Test C	34.3 (9.8)	30.3 (8.8)	26.7 (8.5)	0.018
Color Trail Tests 1	74.3 (36.2)	81.0 (42.3)	88.0 (40.2)	0.323
Color Trail Tests 2	128.6 (57.7)	139.6 (61.6)	159.4 (57.4)	0.054
Postoperative (2 months)				
Digit Span Test	12.6 (2.1)	11.5 (2.4)	11.4 (2.1)	0.864
Digit Symbol Test	37.4 (12.2)	31.4 (11.8)	28.6 (9.7)	0.177
Visual Reproduction Test	9.2 (2.6)	8.5 (2.9)	7.6 (2.7)	0.089
Stroop Color and Word Test A	72.3 (35.1)	75.7 (16.6)	71.5 (15.1)	0.132
Stroop Color and Word Test B	64.3 (14.2)	58.1 (17.0)	52.7 (13.4)	0.046
Stroop Color and Word Test C	35.2 (10.1)	29.7 (9.9)	28.3 (8.4)	0.371
Color Trail Tests 1	124.8 (62.8)	77.1 (37.7)	83.9 (34.7)	0.288
Color Trail Tests 2	81.8 (16.7)	136.7 (65.4)	154.9 (53.0)	0.085

Values are mean (SD).
MMSE, Mini-Mental State Examination.

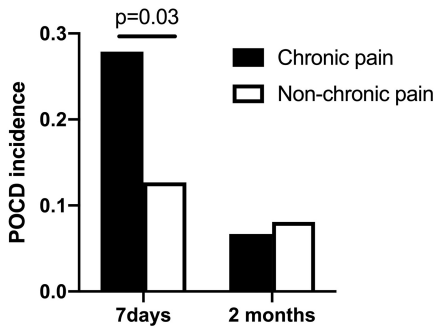

FIGURE 2 | POCD incidence at 7 days and 2 months after surgery (chronic pain patients vs. non-chronic pain patients).

present study is the first to identify chronic preoperative pain as a risk factor of POCD in elderly patients.

Pain of the Patients

The VAS scores indicated that most of the patients without chronic pain had a non-chronic pain lower than grade 3 before surgery while all patients with chronic pain had a grade 3 or 4 preoperative chronic pain. The pain of the patients with chronic pain decreased at 3 days, 7 days, and 2 months after the surgery. As compared with those in patients without chronic pain, the VAS scores were significantly higher in patients with chronic pain at 7 days after surgery ($P < 0.05$) (**Table 3**).

Cognition of the Patients

Higher scores on the Visual Reproduction Test in patients without chronic pain than in patients with chronic pain before surgery indicated that patients with chronic pain had poorer abilities in visual memory. However, regarding the corresponding preoperative cognitive abilities, the scores on of Stroop Color and Word Tests B and C (7 days postoperation) were significantly ($P < 0.05$) higher in patients without chronic pain than those with chronic pain, which indicated that patients with chronic pain had poorer of attention and executive function skills, psychomotor processing speed, and concentration. Many studies have demonstrated that chronic pain impaired memory (Hart et al., 2000; Moriarty et al., 2011; Landro et al., 2013; Herbert et al., 2018). Guusje and colleagues found that high-level pain led to cognitive decline and that elderly adults with chronic pain had

a higher risk of developing cognitive decline (van der Leeuw et al., 2018). Preoperative chronic pain might affect basic physiological functioning of the brain. Eccleston and colleagues (Baliki et al., 2012) adopted the cognitive–affective theory and proposed that the pain experience demands attention and takes precedence over other attention-demanding cognitive processes. Alternatively, in a demonstration of the competing effects of pain on the brain, it has been reported that the distraction of demanding cognitive tasks led to reduced pain intensity and reduced activation of multiple pain-related brain areas in healthy young and middle-aged adults. Thus, it may be that some elderly persons who have chronic pain are unable to draw their attention away from their pain and thereby have difficulty performing cognitive tasks, while others are able to use distraction to manage their pain (Eccleston and Crombez, 1999; Valet et al., 2004).

Risk Factors for Postoperative Cognitive Dysfunction

Risk factors for POCD include advanced age, low education level, preexisting cognitive impairment, major surgery, and general anesthesia. Age is the most substantial risk factor. Although there were differences in the BMI, diagnosis, surgery type, surgery time, and length of hospital stay between the patients with chronic pain and patients without chronic pain, these factors showed no difference between the patients with POCD and patients without POCD (7 days and 2 months after surgery). Since there was no difference in the anesthesia treatment, surgery type, surgery time, blood loss, blood transfusion, and PCIA use between the patients with POCD and the patients without POCD (7 days and 2 months after surgery) in our study, it is still unclear whether generalized anesthesia influences the ability of memory and attention in elderly population.

Many factors might contribute to the easier development of POCD in patients with chronic pain. Although there was no difference in major cognitive functioning between patients with and without chronic pain before surgery, cognitive reserve was lower in the patients with chronic pain (Damoiseaux et al., 2008). This finding might be one of the reasons why POCD developed more easily in the patients with chronic pain. However, another reason might be that patients with chronic pain before surgery experience greater intensity of pain. In a meta-analysis of 29,993 patients who had undergone total knee arthroplasty that exclusively examined preoperative risk factors, preoperative pain was most commonly significantly associated with persistent postsurgical pain (Hah et al., 2019). The present study demonstrated that the VAS scores before surgery and 7 days after surgery were significantly higher in the chronic pain group than in the non-chronic pain group. Compared with the patients without early POCD, the patients who developed POCD had significantly higher VAS scores 3 days after surgery and more often requested additional opioids as rescue analgesia postoperatively. Several studies have demonstrated that postoperative pain was associated with the development of POCD (Marino et al., 2009; Chi et al., 2013; Zywiel et al., 2014). Further research is required to understand how preoperative chronic pain affects POCD.

A systematic review of the influence of anesthesia and pain management indicated that general anesthesia may be associated with early POCD, with no effect seen beyond 7 days after joint arthroplasty (Zywiel et al., 2014). And a meta-analysis of 122 studies revealed that peripheral nerve block anesthesia/analgesia use for patients undergoing primary hip and knee arthroplasty (compared with no use) was associated with lower odds ratios for cognitive dysfunction (odds ratio 0.30, 95% CI 0.17–0.53/odds ratio 0.52, 95% CI 0.34–0.80) (Memtsoudis et al., 2021). Multimodal anesthesia protocols have not been definitively demonstrated to reduce the incidence of POCD. Nonopioid postoperative pain management techniques, limiting narcotics to oral formulations and avoiding morphine, have appeared to reduce the risk of POCD (Zywiel et al., 2014).

Chronic pain is very common in patients who undergo major joint surgery. Since patients with chronic pain before surgery are more likely to develop POCD, more attention should be given to this group of patients. Pain management starting in the preoperative period and high-quality anesthesia should be implemented if possible.

This study had some limitations. Individuals with significant cognitive impairment (MMSE < 24) were excluded from our research cohort. Therefore, our results cannot be generalized to elderly persons with moderate to severe cognitive impairment. We did not assess several other factors, such as low postoperative oxygen saturation and inflammatory mediators in blood. Inflammation plays a central role in osteoarthritis pathogenesis. Those factors potentially could have diluted the effect of preoperative chronic pain on the incidence of POCD. In this study, there were more osteoarthritis patients in the chronic pain group than in the non-chronic pain group. Osteoarthritis is a type of autoimmunity disease; thus, it can potentially affect the occurrence of POCD (Wan et al., 2007; Cibelli et al., 2010; Fidalgo et al., 2011; Terrando et al., 2011; Vacas et al., 2013, 2014). In the present study, we did not evaluate the presence of postoperative delirium. Actually, the data analyzed in this study were from the patients who had finished the second neuropsychological test, which indicated that all patients had no delirium at least by day 7 after surgery.

In conclusion, our study revealed that preoperative chronic pain was associated with an increased risk of early POCD in elderly patients after major joint replacement surgery. High-quality perioperative pain management might be helpful to reduce POCD.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Renji Ethics Committee. The patients/participants

provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

WY, DS, and HX conceptualized the study. YJ, WY, and DS designed the study. XH, LC, HZ, and YF conducted the study and collected the data. XH and XG analyzed the data. XH, XG, and DS drafted the manuscript. All authors revised the manuscript, contributed to the article, and approved the submitted version.

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Gut Microbiome and Plasma Metabolome Signatures in Middle-Aged Mice With Cognitive Dysfunction Induced by Chronic Neuropathic Pain

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Patients with chronic neuropathic pain (CNP) often complain about their terrible memory, especially the speed of information processing. Accumulating evidence suggests a possible link between gut microbiota and pain processing as well as cognitive function *via* the microbiota-gut-brain axis. This study aimed at exploring the fecal microbiome and plasma metabolite profiles in middle-aged spared nerve injury (SNI) mice model with cognitive dysfunction (CD) induced by CNP. The hierarchical cluster analysis of performance in the Morris water maze test was used to classify SNI mice with CD or without CD [i.e., non-CD (NCD)] phenotype. 16S rRNA sequencing revealed a lower diversity of gut bacteria in SNI mice, and the increase of *Actinobacteria*, *Proteus*, and *Bifidobacterium* might contribute to the cognitive impairment in the CNP condition. The plasma metabolome analysis showed that the endocannabinoid (eCB) system, disturbances of lipids, and amino acid metabolism might be the dominant signatures of CD mice. The fecal microbiota transplantation of the Sham (not CD) group improved allodynia and cognitive performance in pseudo-germ-free mice *via* normalizing the mRNA expression of eCB receptors, such as *cn1r*, *cn2r*, and *htr1a*, reflecting the effects of gut bacteria on metabolic activity. Collectively, the findings of this study suggest that the modulation of gut microbiota and eCB signaling may serve as therapeutic targets for cognitive deficits in patients with CNP.

Keywords: pain, cognitive dysfunction, gut microbiome, metabolites, endocannabinoids

INTRODUCTION

Accumulating preclinical and clinical evidence suggests that cognitive impairment is a common comorbidity of chronic neuropathic pain (CNP) (Attal et al., 2014; Mazza et al., 2018; Fonseca-Rodrigues et al., 2021; Rouch et al., 2021). On the one hand, neuropathic pain results in worse cognitive performance, whereas on the other hand, cognitive deficits also affect the perception of pain (Fonseca-Rodrigues et al., 2021). Previous studies have proposed several potential mechanisms about the comorbidity, such as limited attention capacity, brain structural changes, altered

neurotransmitters, receptors, and neural mediators (e.g., enzymes, neurotrophic factors, and cytokines) (Moriarty et al., 2011; Mazza et al., 2018). However, the mechanisms underlying cognitive impairment associated with CNP have not yet been elucidated. Considering the fact that CNP and cognitive impairment are refractory to current pharmacological agents, specific targets for indicating or preventing the comorbidity are urgently needed.

Several studies have proposed that gut microbiota play critical roles in several central nervous system (CNS)-related conditions, such as pain and cognition (Amaral et al., 2008; Cryan and Dinan, 2012; Shen et al., 2017; Guo et al., 2019). Recent evidence suggests that dysbiosis exists in several types of CNP and cognitive impairments (Jiang et al., 2017; Lin et al., 2020; Marttinen et al., 2020). Moreover, the interventions of gut microbiota, such as probiotics and fecal microbiota transplantation (FMT), showed beneficial effects on the two disorders (Van Laar et al., 2019; Bonomo et al., 2020; Cuozzo et al., 2021). Studies have also revealed that gut microbiota regulates the development of CNP or cognitive impairment through the vagus nerve, endocrine, and metabolic as well as immune communications (Sampson and Mazmanian, 2015; Sun et al., 2020; Chen et al., 2021). However, it is not known how the microbiota affects the comorbidity.

Interactions between the host and microbiota cause a wide fluctuation in the circulating neuroactive substances, such as neurotransmitters, short-chain fatty acids, and bile acids (Cryan and Dinan, 2012; Sharon et al., 2016; Li et al., 2020). It is worth noting that the disturbance of the circulating metabolites is one of the major routes through which microbiota affect the brain. Therefore, this calls for the combinative analyses of the microbiome and plasma metabolome, which will confirm the microbial signatures and related neural pathways. In this study, mice with cognitive impairment phenotype were screened in a spared nerve injury (SNI) CNP model based on the behavioral results. We explored the alterations of gut microbiota and plasma metabolites by performing 16S rRNA sequencing and non-targeted metabolomics, which is the overarching goal of identifying potential biomarkers for predicting the comorbidity of CNP and cognitive impairment. Furthermore, the relationship between abnormal bacteria and metabolites was analyzed to evaluate the possible metabolic pathways involved in the comorbidity. Finally, pseudo-germ-free (pGF) mice were constructed to investigate the effects of FMT on endocannabinoid (eCB) signaling and pain as well as cognition.

MATERIALS AND METHODS

Animals

Of note, 2- or 10-month-old male C57BL/6 mice in this study were obtained from the Animal Center of Tongji Hospital. In total, 71 mice were enrolled and randomly divided into different groups. Five mice were housed in an individual ventilated cage at 22 ± 2°C and 12-h light/dark cycles (lights on 8:00 a.m.) and with *ad libitum* food and water. Animals were acclimated to the environmental conditions for 7 days before the experiment. Procedures for the animal experiments were in accordance with the ethical guidelines of the National Institutes of Health and the International Association for the Study of Pain. This study was approved by the Experimental Animal Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Notably, efforts were made to minimize animal suffering and the number of animals used.

Establishment of SNI Mice Model

A previous study found that middle-aged (i.e., 10 months old) rodents were more susceptible to cognitive impairment related to chronic pain compared to young (i.e., 3 months old) and old (i.e., 22 months old) groups (Leite-Almeida et al., 2009). Thus, middle-aged mice were used to establish the cognitive impairment model induced by CNP in this study. Mice were anesthetized with 3% isoflurane for induction and 1.5% isoflurane for maintenance. After dissecting the skin and the musculature in the surgical site on the left limb lateral surface, the sciatic nerve and its three terminal branches (i.e., sural, common peroneal, and tibial nerves) were exposed. Then, the common peroneal and tibial nerves were tightly ligated with 4–0 silk ligatures and dissected the distal to the ligation. The mice were placed on a heating blanket to keep warm during the procedure. After suturing the skin and muscles, the mice were returned to their previous cages. Mice in the Sham group also underwent similar procedures, but the nerves were only exposed without touching.

Open Field Test

The open field test (OFT) was used to test the effect of SNI surgery on motor activities. After being habituated to the environment for 30 min, animals were placed at the center of the Plexiglas chamber (40 × 40 × 40 cm) to explore freely for 5 min, and the total distance traveled (in centimeters) was automatically monitored and analyzed using the automatic tracking system (ZhongShi technology, Beijing, China). The inner floor and wall of the chamber were cleaned with 3% acetic acid in the interval.

Mechanical Withdraw Threshold

The mechanical allodynic response was used as a hall marker for CNP in SNI mice. Mice were placed in separate Plexiglas light-transparent chambers on the elevated mesh wire for 30 min before the test to habituate to the examination condition. The von Frey filaments (0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, and 1 g) were applied to the lateral surface (sural nerve territory) on the left foot for 6 s and repeated five consecutive times. The procedure started with the lowest force (0.008 g) and progressively increased to higher von Frey filaments until positive responses occurred in the experiment animal. Notably, quick paw withdrawal or licking was considered a positive pain-like response. The next lower filament was applied when a positive response was observed at least three times in five consecutive times under the same force. It should be noted that the next higher filament was also used when no positive response occurred. The lowest filament (in grams) that can induce a positive response was recorded as the MWT.

Morris Water Maze Test

The *Morris water maze test* (MWMT), consisting of a 5-day training and a probe test on the 6th day, was performed to

evaluate the spatial reference memory according to our previous studies (Zhan et al., 2018, 2019). Mice were trained to locate the 10-cm-diameter hidden platform (submerged 0.5–1 cm below the water surface) three times per day for 5 consecutive days in a circular pool (diameter 120 cm, height 50 cm) filled with opaque water (23 ± 1°C). After locating the hidden platform, mice were allowed to stay there for 15 s. Mice that failed to find the hidden platform within 60 s were gently guided to the platform and allowed to remain there for 15 s. The platform was placed at the same location throughout the 5-day training test. The time taken to locate the platform (escape latency) was recorded using a digital camera (ZhongShi Technology, Beijing, China) and used to evaluate the spatial learning ability of the mice. A probe test was then performed on the 6th day to evaluate the spatial memory capacity of the animal. After the 5-day training, the hidden platform was removed from the pool, and mice were given 60 s to swim freely in the pool. The number of crossing times and time spent in the target quadrant were automatically recorded.

16S rRNA Sequencing of Fecal Samples

Total fecal genomic DNA was extracted using a DNA Extraction Kit (Qiagen Inc., Valencia, CA, USA) according to the instructions of the manufacturer. The concentration of DNA was then determined using NanoDrop (NanoDrop Technologies Inc., DE, USA) and agarose gel electrophoresis. The extracted genomic DNA was used as a template for PCR amplification using the barcoded primers and Tks Gflex DNA Polymerase (Takara Biomedicals, Beijing, China). For the bacterial diversity analysis, V3–V4 variable regions of 16S rRNA genes were amplified using universal primers: 343 F (5′-TACGGRAGGCAGCAG-3′) and 798 R (5′-AGGGTATCTAATCCT-3′). Raw sequencing data were then spliced using the sliding window trimming approach to cut off ambiguous bases and low-quality sequences. Then, further denoising was performed on the sequences using the QIIME software, and reads with 75% of bases above Q20 were retained. Finally, operational taxonomic units and clustering were performed using the VSEARCH software, with 97% similarity as the cutoff.

Liquid Chromatography-Mass Spectrometry Analysis of Plasma Metabolites

Plasma samples, which had been stored at −80°C, were thawed in an ice bath before the process. The metabolites were extracted as previously described (Braundmeier-Fleming et al., 2016; Song et al., 2021). In brief, 20 μl of the sample was dissolved in an extraction solution consisting of 2-chloro-l-phenylalanine, methanol, and acetonitrile, followed by centrifugation at 13,000 rpm for 15 min at 4°C. The supernatant was then transferred into a brown glass vial and dried in a freeze concentration centrifugal dryer. Each sample was homogenized using a mixture of methanol and water (1/4 v/v) and centrifuged at 13,000 rpm for 5 min at 4°C. The supernatants were collected in glass vials and stored at −80°C until the liquid chromatography-mass spectrometry (LC-MS) analysis. After sample preparation, plasma metabolites were identified and quantified by ACQUITY UPLC I-Class system (Waters Corporation, Milford, CT, USA) and VION IMS QTOF Mass Spectrometer (Waters Corporation, Milford, CT, USA) in both positive and negative ion modes. Gradient elution (mobile A: water with 0.1% formic acid; mobile B: Acetonitrile/Methanol with 0.1% formic acid) was performed to separate the samples on an ACE C18 column (1.7 μm, 2.1 × 100 mm) at a flow rate of 0.4 ml/min. The linear gradient was as follows: 0 min, 1% B; 1 min, 30% B; 2.5 min, 60% B; 6.5 min, 90% B; 8.5 min, 100% B; 10.7 min, 100% B; 10.8 min, 1% B; and 13 min, 1% B. Finally, the acquired raw data were analyzed using the progenesis QI software (Waters Corporation, Milford, CT, USA).

pGF Mice Modeling

The pGF mice were established according to our previous studies (Zhan et al., 2018; Yang et al., 2019). In brief, broad-spectrum antibiotics were dissolved in drinking water in the following concentrations: 1 g/L ampicillin, 1 g/L neomycin sulfate, and 1 g/L metronidazole (Sigma–Aldrich, Shanghai, China). Mice were exposed to plain water or water with antibiotics for 14 days. Notably, the drinking solution was replenished every 2 days.

Fecal Microbiota Transplantation

Fresh feces were collected from anal orifices of donors and immediately used to prepare the microbiota solution. The solution was prepared by diluting 1 g of fresh feces into 10 ml sterile phosphate-buffered saline (PBS), followed by a vortex to suspend the microbiota. After antibiotics treatment, pGF mice were given 0.2 ml PBS or fecal microbiota suspension obtained from the Sham and cognitive dysfunction (CD) group mice by oral gavage once a day for 14 consecutive days.

Measurement of eCB Signaling in the Prefrontal Cortex Tissues Using Quantitative PCR

Total RNA was extracted from prefrontal cortex (PFC) tissues using TRIzol reagent (Takara Biomedicals, Beijing, China) in accordance with the instructions of the manufacturer. In brief, tissues were immersed in 500 μl TRIzol reagent and then homogenized on ice with a homogenizer. Then, chloroform (100 μl) was added to the suspension, followed by mixing and centrifugation to isolate the aqueous phase with RNA. Subsequently, RNA was precipitated with a volume of isopropyl alcohol and washed with 75% ethanol. The concentration and purity of RNA suspended with diethylpyrocarbonate (DEPC)-treated water was evaluated using a NanoDrop (NanoDrop Technologies Inc., DE, USA) according to the absorbance at 260 (*A260*) and 280 nm (*A280*). Notably, each sample was measured three times to ensure the precision of measurements. Then, cDNA was synthesized from 1 μg of RNA using a cDNA Synthesis Kit (Vazyme, Nanjing, China) according to the protocol of the manufacture and then used for the quantitative PCR (qPCR) analysis. Primers of the target gene for the eCB system were designed using Primer-Blast and are listed in **Supplementary Table 1**. Each sample was run in duplicate in a 20-μl reaction, including 0.5 μl forward and reverse primers, 20 ng of cDNA, and 10 μl SYBR Green Master Mix (Vazyme,

Nanjing, China). The following reaction conditions were used for RNA amplification: 95°C for 5 min, followed by 45 cycles of denaturing 20 s at 95°C, annealing 30″ at 70°C, and extension 30″ at 72°C. Relative gene expression was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels and quantified using the ΔΔCt method. The mean relative expression level of each gene in the control group was standardized to 1 to facilitate the comparison. The results were presented as fold change relative to the control group.

Statistical Analysis

All analyses were performed using GraphPad Prism software version 8.0, and all data were presented as the mean ± SEM. Comparisons among groups were performed using the one-way ANOVA or two-way ANOVA, followed by Tukey's *post hoc* test. For different time points, two-way repeated-measures (RM) ANOVA was performed to compare the difference among groups. Discontinuous data, such as the platform crossing times of the animal in the probe test, and sequencing data not conforming to the normal distribution were analyzed using Kruskal–Wallis non-parametric test, followed by Dunn's *post hoc* test. In hierarchical cluster analysis, the data were first standardized using *z*-scores. Then, the hierarchical cluster analysis of escape latency, platform crossing times, and time spent in the target quadrant was performed using Ward's method. Squared Euclidean distance was applied as the distance measure, and mice were classified as CD or non-CD (NCD) clusters. Finally, Spearman's correlation was performed to analyze the correlation between gut microbiota and plasma metabolites. $P < 0.05$ was considered statistically significant.

RESULTS

SNI-Induced CNP Impaired Spatial Reference Memory in Middle-Aged Mice

A total of 31 mice underwent SNI to develop neuropathic pain (**Figure 1A**). Mechanical allodynia was observed on the 7th post-SNI and persisted until the end of the experiment (day 28) (**Figure 1C**). After 1 month, SNI mice were divided into CD (54.8%) group or NCD (45.2%) group according to the hierarchical clustering analysis of the MWMT performance (**Figure 1B**). The representative trace graphs of the three groups in the probe trial were presented in **Figure 1D**. OFT was performed before MWMT to evaluate the locomotor activity, and the total distance traveled in the open field showed no significant difference among the groups, suggesting that the locomotor ability was not compromised by SNI (**Figure 1E**). In addition, MWMT was used to evaluate reference memory among the groups. Results showed that the CD mice exhibited significantly higher escape latency in the training test compared to the Sham and NCD mice, indicating the impairment learning capacity (**Figure 1F**). In the probe trial, the time spent in the target quadrant and the numbers of platform crossing were significantly decreased in the CD mice compared to the Sham and NCD mice (**Figures 1G,H**). These results suggest that the hierarchical clustering analysis is an effective approach to discriminate CD mice after SNI surgery. Excluding the mice without cognitive impairment from the SNI group facilitates us to find specific associations with CNP-related CD (in this case, gut microbiota and plasma metabolites).

Differential Gut Microbiota Profiling Among the Sham, CD, and NCD Mice

The 16S rRNA sequencing was performed to explore the alterations in fecal samples from the Sham, CD, and NCD groups. Simpson's Index, an indicator of the α-diversity of gut microbiota, was significantly increased in the CD and NCD groups compared to the Sham group, suggesting lower diversity of species and bacteria after SNI surgery (**Figure 2A**). Notably, there was no significant change between the CD and NCD groups with regard to the Simpson's Index. Then, the principal component analysis (PCA) and the principal coordinate analysis (PCoA) were used to evaluate the β-diversity, which reflects the similarity and dissimilarity of sample community composition. According to the obtained results, the Sham group clustered far away from the CD and NCD groups, while there was no significant difference between the CD and NCD groups (**Figures 2B,C**). The heatmap of the gut microbiota composition at different levels exhibited specific differences among the three groups. **Figure 2D** shows the top 15 dominant microflora in samples at the phylum and genus level, whereas **Figures 3A,B** show the top 10 significantly differential microbes among the three groups at phylum and genus levels, respectively.

Then, the linear discriminant analysis (LDA) combined with effect size measurements (LEfSe) were used to identify the differentially abundant taxa in the Sham, CD, and NCD groups. Gut bacteria that were enriched in the CD group (and thus depleted in the Sham and NCD groups) included *Proteus* (a member in the *Proteobacteria* phylum) and *Actinobacteria*. Six dominant bacteria were present in the Sham and NCD groups, respectively. In contrast, *Bacteroidaceae*, *Bacteroides*, *Citrobacter*, *Hungatella*, *Clostridioides*, and *Uncultured_bacterium* were higher in the Sham group as compared to the CD and NCD groups. Interestingly, most of the abundant taxa in the Sham and NCD groups were from *Firmicutes* and *Bacteroidetes* phyla, with the exception of *Citrobacter* and *Uncultured_bacterium* (**Figure 3C**).

Furthermore, the results revealed that a total of five gut bacteria were significantly increased in the CD mice compared to those in the Sham and NCD groups (**Figures 3D–H**). It is worth noting that *Nonomuraea*, and *Libanicoccus* were only detected in CD mice, suggesting their promising diagnostic role in patients with CD comorbid with chronic pain, although further studies are required for validation.

Alterations in Plasma Metabolic Profile Among the Sham, CD, and NCD Mice

Untargeted LC-MS metabolomic profiling identified 101 different metabolites with variable importance in the projection (VIP) scores larger than 1 and $P < 0.05$ as the cutoff. To elucidate on the roles of the differential metabolites, we queried and filtered them in the Human Metabolome Database (HMDB), the Small Molecule Pathway Database (SMPDB),

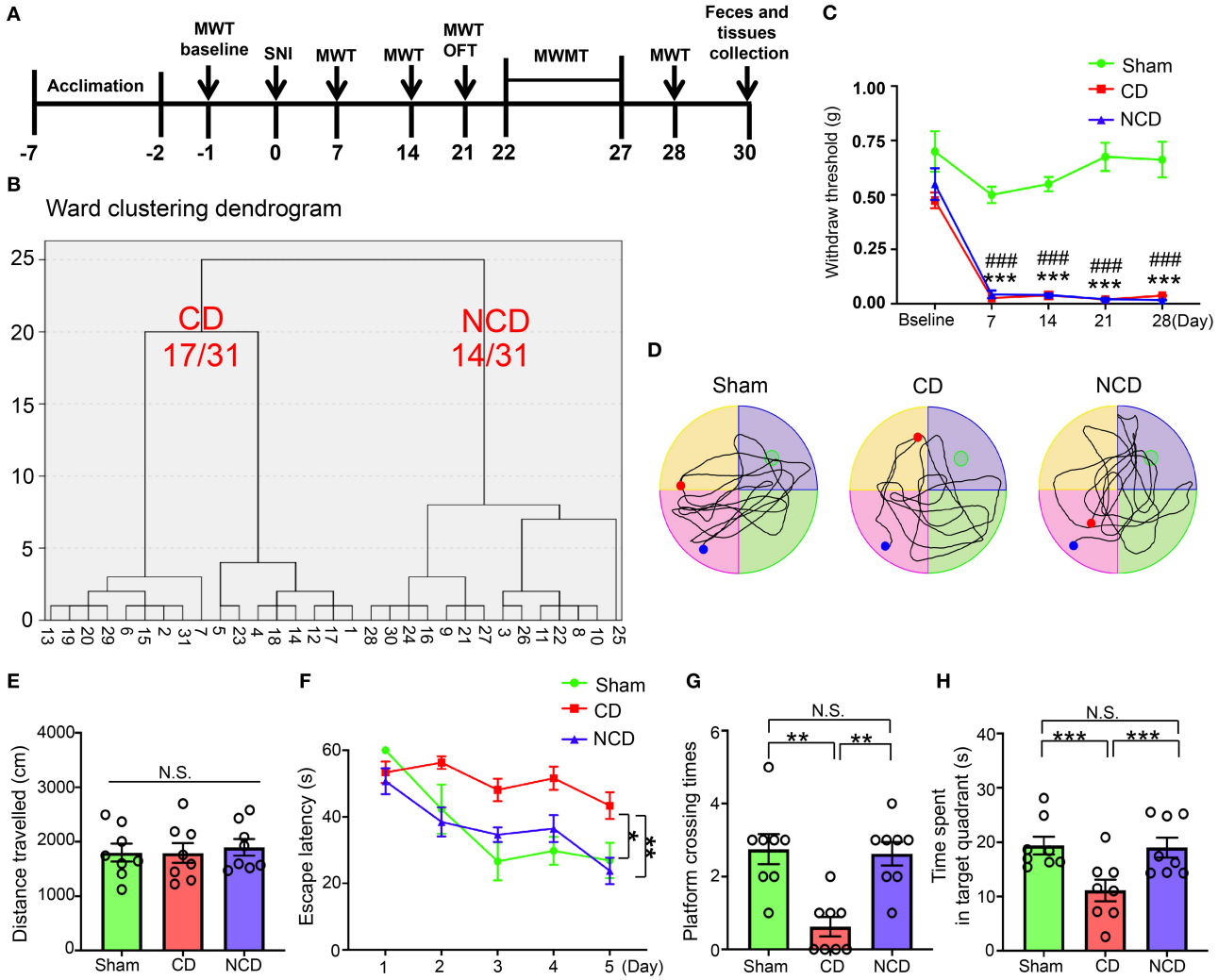

FIGURE 1 | Spatial learning and reference memory were impaired in CD mice. **(A)** The schedule of the experiment. SNI was performed on day 0 after acclimation. MWT was measured on day 1 before the surgery and on day 7, 14, 21, and 28 after SNI. From day 22–27 after the surgery, MWMT was applied to evaluate the spatial reference memory of animals in Sham and SNI groups. Tissues and fecal samples were collected on day 30 post-SNI. **(B)** MWT [two-way repeated-measures (RM) ANOVA; Time: $F_{(4, 84)} = 39.63$, $P < 0.001$; Group: $F_{(2, 21)} = 197.9$, $P < 0.001$; Interaction: $F_{(8, 84)} = 6.184$, $P < 0.001$]. **(C)** Dendrogram of hierarchical clustering analysis. A total of 31 mice after SNI were divided into CD and NCD groups by the escape latency, platform crossing, and time spent in the target quadrant results of hierarchical clustering analysis. Of note, 17 of 31 mice (CD group) exhibited worse performance in the spatial learning and memory functions, including the latency to reach the target platform, the times of crossing the platform, and the time spent in the target quadrant. **(D)** The graphs of the representative traces of Sham, CD, and NCD groups in the probe test. **(E)** Total distance in OFT [one-way ANOVA; $F_{(2, 21)} = 0.124$, $P > 0.05$]. **(F)** The latency to reach the submerged platform in MWMT [two-way RM ANOVA; Time: $F_{(4, 84)} = 12.38$, $P < 0.001$; Group: $F_{(2, 21)} = 43.37$, $P < 0.001$; Interaction: $F_{(8, 84)} = 1.992$, $P = 0.057$]. **(G)** Crossing times in the probe test [Kruskal–Wallis non-parametric test; $H_{(2, 21)} = 12.94$; $P = 0.002$]. **(H)** The time spent in the target quadrant [one-way ANOVA; $F_{(2, 21)} = 6.5$, $P = 0.006$]. Data are presented as means ± SEM ($n = 8$ per group) and analyzed by one-way ANOVA or two-way ANOVA followed by Tukey's *post hoc* test or Kruskal–Wallis non-parametric test followed by Dunn's *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, CD vs. Sham. $^{\#\#\#}P < 0.001$, NCD vs. Sham. CD, cognitive dysfunction; SNI, spare nerve injury; MWMT, Morris water maze test; MWT, mechanical withdraw threshold; NCD, non-cognitive dysfunction; N.S., not significant; OFT, open field test.

and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Notably, only 61 of these 101 metabolites had tentative IDs in the abovementioned databases (details listed in **Supplementary Table 2**).

The PCA results revealed that the metabolomic profiles differed among the Sham, CD, and NCD groups (**Figure 4A**), suggesting a pivotal role of plasma metabolites in CNP-induced CD. However, the partial least squares discriminant analysis (PLS-DA) failed to separate the CD group from the NCD group completely (**Figure 4B**). Then, the KEGG pathway enrichment analysis was performed to explore the functions of the changed metabolites in CNP-induced CD mice. Results revealed that the most enriched KEGG pathways were "Retrograde eCB signaling," "Glycosylphosphatidylinositol (GPI)-anchor biosynthesis,"

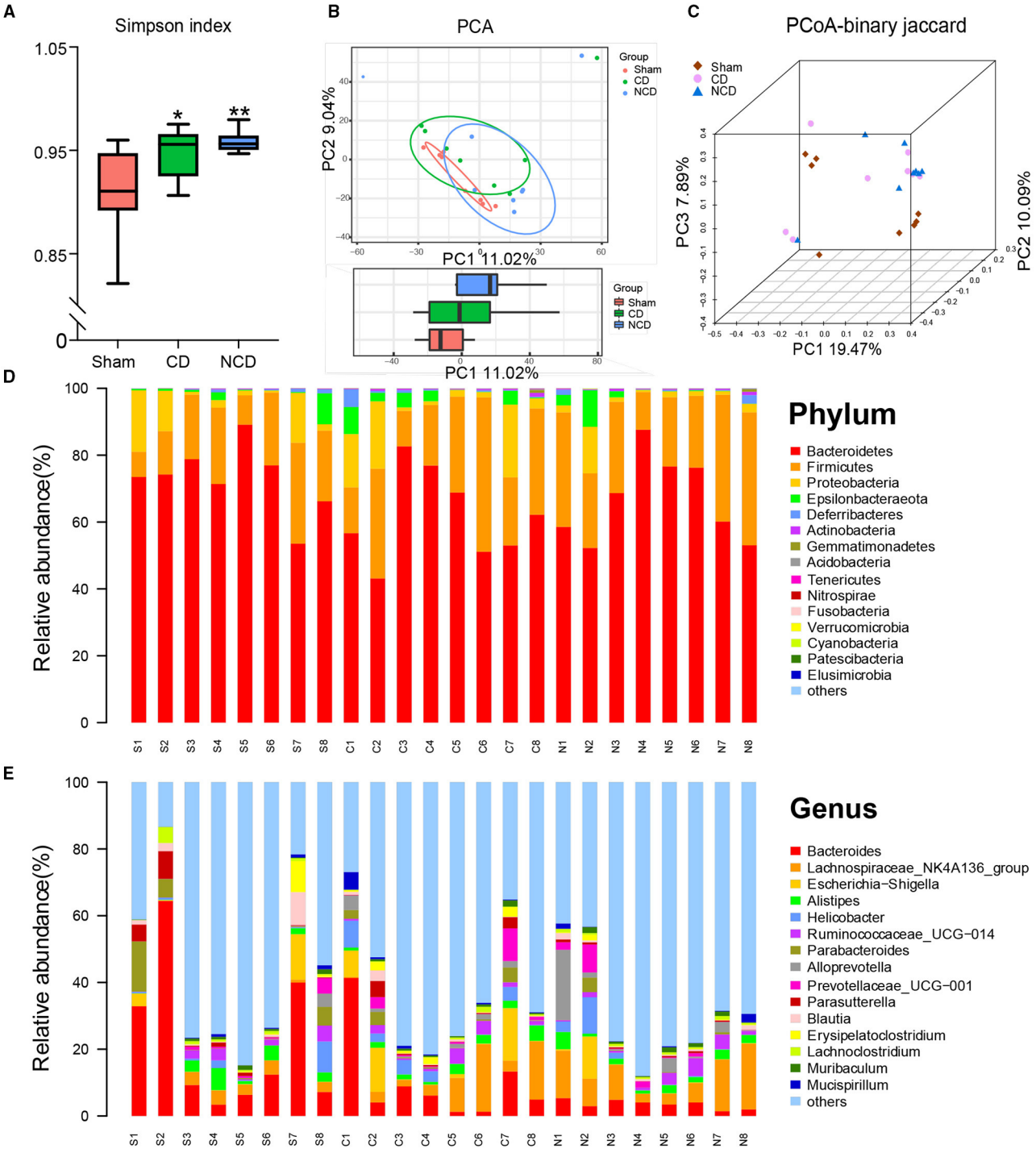

FIGURE 2 | Differential profiles of gut microbiota among Sham, CD, and NCD groups. **(A)** Simpson index [$F_{(2,\ 21)} = 6.557$, $P = 0.006$]. **(B)** The PCA of gut microbiota (PC1 vs. PC2). **(C)** PCoA-binary Jaccard dissimilarity. **(D)** The top 15 abundant flora among the groups at the phylum level. **(E)** The top 15 abundant flora among the groups at the genus level. Data are presented as means ± SEM ($n = 8$ per group) and analyzed by one-way ANOVA followed by Tukey's *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$. CD, cognitive dysfunction; NCD, non-cognitive dysfunction; N.S., not significant.

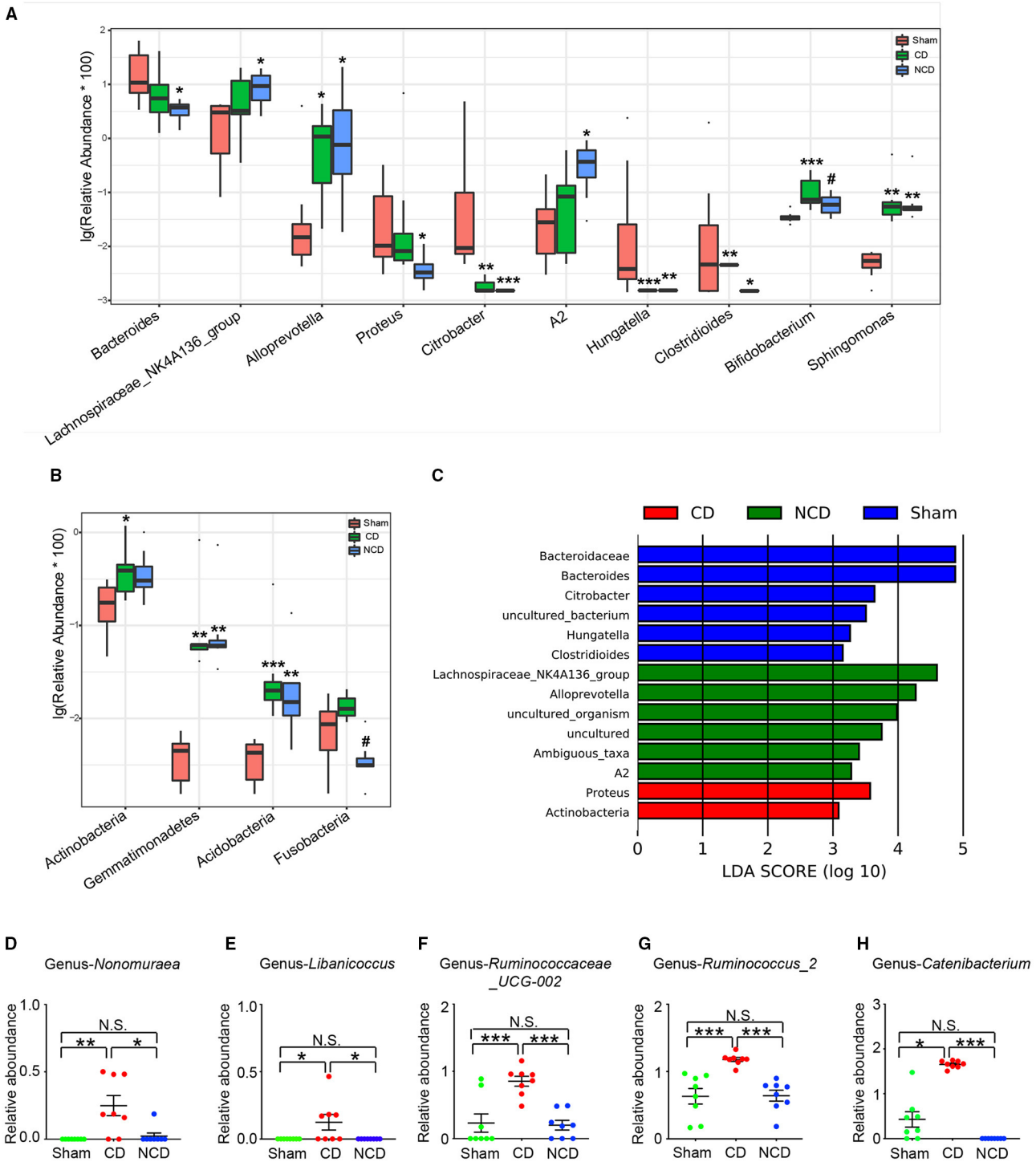

FIGURE 3 | Microbiota with significant changes at phylum and genus levels among the groups. **(A)** The top 10 flora with significant difference among the groups at genus level: *Bacteroides* [Kruskal–Wallis non-parametric test, $H_{(2,\ 21)} = 8.205$; $P = 0.02$]; *Lachnospiraceae-NK4A136-group* [Kruskal–Wallis non-parametric test, $H_{(2,\ 21)} = 6.14$; $P = 0.046$]; *Alloprevotella* [Kruskal–Wallis non-parametric test, $H_{(2,\ 21)} = 8.315$; $P = 0.02$]; *Proteus* [Kruskal–Wallis non-parametric test, $H_{(2,\ 21)} = 8.05$; $P = 0.02$]; *Citrobacter* [Kruskal–Wallis non-parametric test, $H_{(2,\ 21)} = 17.92$; $P = 0.0001$]; *A2* [Kruskal–Wallis non-parametric test, $H_{(2,\ 21)} = 7.985$; $P = 0.02$]; *Hungatella* [Kruskal–Wallis non-parametric test, $H_{(2,\ 21)} = 15.91$; $P = 0.0004$]; *Clostridioides* [Kruskal–Wallis non-parametric test, $H_{(2,\ 21)} = 10.64$; $P = 0.005$]; *Bifidobacterium* [Kruskal–Wallis non-parametric test, $H_{(2,\ 21)} = 11.33$; $P = 0.0035$]; *Sphingomonas* [Kruskal–Wallis non-parametric test, $H_{(2,\ 21)} = 15.44$; $P = 0.0004$]. **(B)** The phylum

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FIGURE 3 | with significant difference among the groups: *Actinobacteria* [Kruskal–Wallis non-parametric test, $H_{(2,\ 21)} = 6.995$; $P = 0.0008$]; *Gemmatimonadetes* [Kruskal–Wallis non-parametric test, $H_{(2,\ 21)} = 15.38$; $P = 0.0005$]; *Acidobacteria* [Kruskal–Wallis non-parametric test, $H_{(2,\ 21)} = 14.41$; $P = 0.0007$]; *Fusobacteria* [Kruskal–Wallis non-parametric test, $H_{(2,\ 21)} = 12.56$; $P = 0.002$]. **(C)** Taxonomic groups showing linear discriminant analysis (LDA) scores > 3.0. **(D)** Significant altered gut bacteria in CD mice compared with Sham and NCD mice. *Nonomuraea* [Kruskal–Wallis non-parametric test, $H_{(2,\ 21)} = 11.77$; $P = 0.003$]; *Libanicoccus* [Kruskal–Wallis non-parametric test, $H_{(2,\ 21)} = 9.105$; $P = 0.01$]; *Ruminococcaceae_UCG-002* [one-way ANOVA, $F_{(2,\ 21)} = 14.13$; $P < 0.001$]; *Ruminococcus_2* [one-way ANOVA, $F_{(2,\ 21)} = 14.19$; $P < 0.001$]; *Catenibacterium* [Kruskal–Wallis non-parametric test, $H_{(2,\ 21)} = 19.65$; $P < 0.001$]. Data are presented as means ± SEM ($n = 8$ per group). The data that passed the normality and lognormality test were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. Otherwise, Kruskal–Wallis non-parametric test followed by Dunn's *post hoc* test was used. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with Sham. $^{\#}P < 0.05$ compared with CD. CD, cognitive dysfunction; NCD, non-cognitive dysfunction; N.S., not significant.

"Autophagy-other," and "Glycerophospholipid metabolism" (**Figures 4C,D**). These enriched pathways provided novel insights into the pathogenic mechanism of CNP-induced CD, which should be the subject of future research.

Metabolites involved in the retrograde eCB signaling pathway included Anandamide (20: l, n-9)/N-arachidonoylethanolamine (AEA), N-acetylornithine, and N-undecanoylglycine (**Figures 4L–N**). The relative abundance of the three eCB metabolites presented a downward trend in both CD and NCD mice, suggesting that chronic pain had a greater effect on the plasma metabolites compared with cognitive disorder. With the exception of Nb-palmitoyltryptamine, tryptophan-related metabolites, such as docosahexaenoyl serotonin (DHA-5-HT), Trp-P-1, and indoleacetaldehyde, were significantly decreased in mice with CD (**Figures 4E,G–I**). In contrast, the relative abundances of L-alloisoleucine, glutathione, and allocholic acid were significantly increased in the CD group compared to the Sham and NCD groups (**Figures 4F,J,K**).

Correlations Between Gut Microbiota and Plasma Metabolites

Spearman's correlation coefficient was applied to evaluate the connection between gut microbes and plasma metabolites (**Figure 5**). Results showed that *Oribacterium* and *Bacteroides* were strongly correlated with pain-associated metabolites. For example, *Oribacterium* was significantly positively correlated with pyroglutamic acid, estrone, 2-methoxyestradiol, and lysyl-glycine. Significant negative correlations were found with deoxycholic acid, 3-methylpentanoic acid, and lithocholic acid. Interestingly, the same correlation was found between *Bacteroides* and the abovementioned metabolites. In addition, deoxycholic acid was negatively correlated with most microbes, such as *Oribacterium*, *Oscillibacter*, and *Bacteroides*. However, these microbes were positively correlated with the level of lysyl-glycine. Deoxycholic acid and lysyl-glycine are bile acids and dipeptides, respectively. Moreover, they have been reported to exert physiological or cell-signaling effects on the microbiota-gut-brain axis (Li et al., 2020; Ahn et al., 2021). Collectively, these results suggest that the decreased *Bacteroides*- and *Oribacterium*-related deoxycholic acid accumulation, as well as lysyl-glycine deficiency, might undermine chronic pain-induced CD.

Effects of FMT on Nociception and Reference Memory in pGF Mice

To investigate whether changes in spatial learning and memory performance in the SNI mice were caused by dysbiosis, pGF mice were established by the oral administration of broad-spectrum antibiotics at large doses for 14 consecutive days. After treatment with antibiotics, gut microbiota from the Sham or CD mice were transplanted into pGF mice for 2 weeks (**Figure 6A**). The body weight of pGF mice was significantly lower 1 week after antibiotics treatment compared to the control mice but returned to baseline by the end of the antibiotics treatment (day 14). There was no significant difference in body weight among the control, pGF mice receiving PBS, Sham, and CD mice on 21st-day post-antibiotics treatment. However, body weight was significantly lower in the mice receiving Sham and CD fecal transplantation than in the vehicle control mice on the 28th-day posttreatment (**Figure 6B**). These findings suggested that gut microbiota might influence the metabolism in the host mice. However, food intake during antibiotic treatment should be noticed. There was no difference in MWT among the groups at baseline, but the repeated administration of antibiotics undermined the MWT scores. Interestingly, the FMT from the Sham mice, but not from the CD group mice, significantly restored the allodynia phenotype caused by antibiotics treatment (**Figure 6C**).

Changes in the total distance traveled in OFT were not different among the four groups before the MWMT (**Figure 6E**). The representative trace graphs of each group in the probe trial were presented in **Figure 6D**. Spatial learning and memory performances (including escape latency, platform crossing times, and time spent in the target quadrant) were worse in the PBS group compared to the control group. Furthermore, the transplantation of gut microbiota from the Sham mice, but not from the CD mice, effectively improved the poor performance in the pGF mice (**Figures 6F–H**). Overall, these results suggest that the behavioral performance was influenced by host microbiota composition.

Fecal Microbiota From the Sham Mice Normalized PFC *Cnr1*, *Cnr2*, and *Htr1a* MRNA Expression Caused by Antibiotic Treatment

To explore the role of eCB signaling in the interaction of dysbiosis and cognition as well as nociception, we measured the mRNA levels of classic cannabinoid receptors (*cn1r* and *cn2r*) and non-cannabinoid receptors, such as peroxisome proliferator-activated receptor alpha (*ppara*), G protein-coupled receptor (GPR) 55 (*gpr55*), *gpr119*, transient receptor potential action channel, subfamily V, member 1 (*trpv1*), and serotonin$_{1A}$ receptor (*htr1a*). It should be noted that these receptors are activated directly

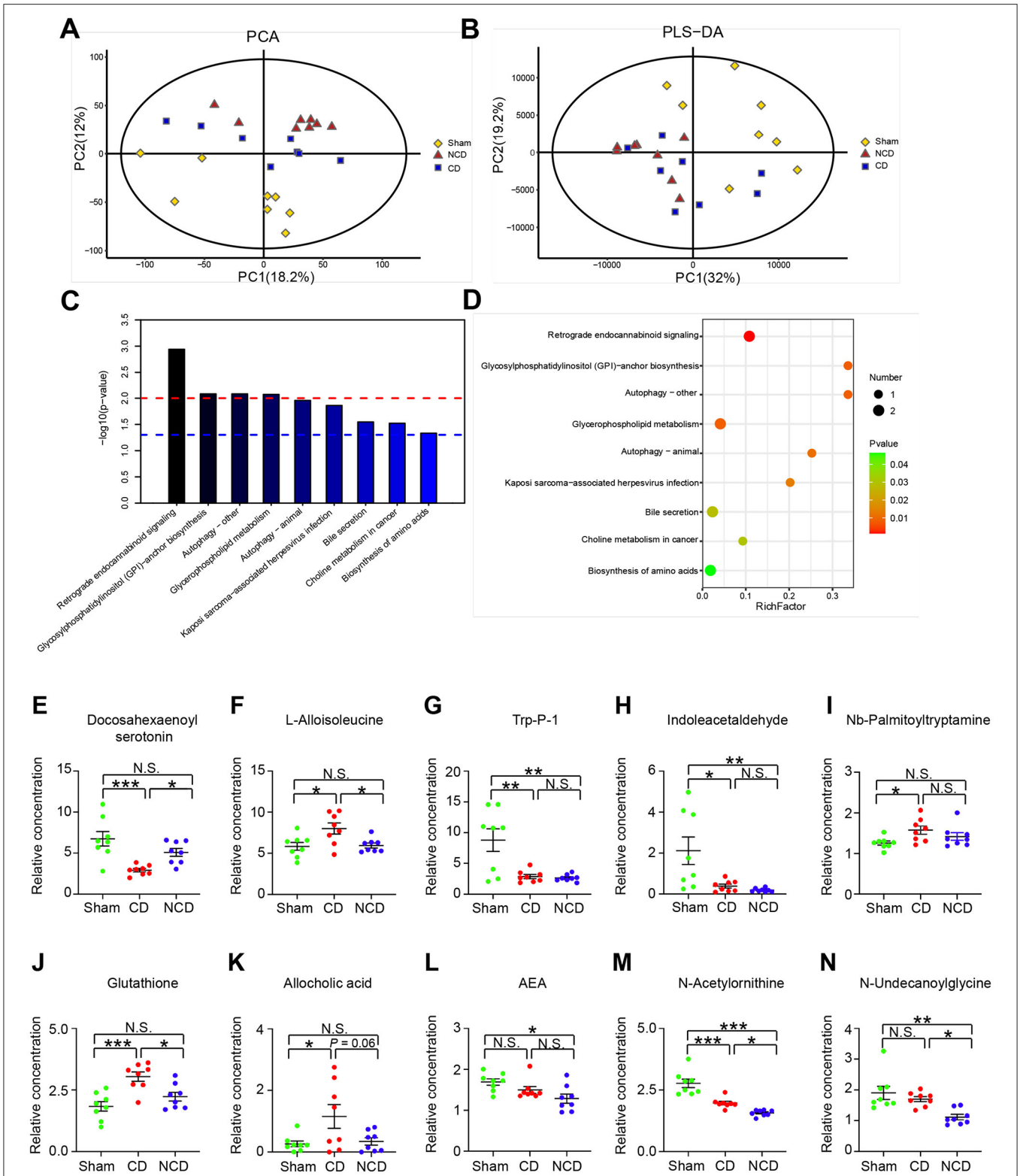

FIGURE 4 | Metabolic profiling analysis among the groups. **(A)** Principal component analysis (PCA). **(B)** The partial least squares discriminant analysis (PLS-DA) of plasma metabolites. **(C)** The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of differentially expressed metabolites. Y-axis shows the *P*-value of a particular pathway. **(D)** Enriched KEGG pathways in plasma metabolites. Rich factor indicates the ratio of differentially expressed metabolites to the total metabolites

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FIGURE 4 | number in a certain pathway. **(E)** Docosahexaenoyl serotonin [one-way ANOVA; $F_{(2,\ 21)} = 10.3$, $P < 0.001$]; **(F)** L-Alloisoleucine [one-way ANOVA; $F_{(2,\ 21)} = 5.84$, $P = 0.009$]; **(G)** Trp-P-1 [one-way ANOVA; $F_{(2,\ 21)} = 10.49$, $P < 0.001$]; **(H)** Indoleacetaldehyde [one-way ANOVA; $F_{(2,\ 21)} = 7.259$, $P = 0.004$]; **(I)** Nb-Palmitoyltryptamine [one-way ANOVA; $F_{(2,\ 21)} = 3.57$, $P = 0.05$]; **(J)** Glutathione [one-way ANOVA; $F_{(2,\ 21)} = 10.81$, $P < 0.001$]; **(K)** Allocholic acid [one-way ANOVA; $F_{(2,\ 21)} = 4.24$, $P = 0.028$]; **(L)** N-Arachidonoylethanolamine [one-way ANOVA; $F_{(2,\ 21)} = 8.015$, $P = 0.018$]; **(M)** N-Acetylornithine [one-way ANOVA; $F_{(2,\ 21)} = 30.86$, $P < 0.001$]; **(N)** N-Undecanoylglycine [one-way ANOVA; $F_{(2,\ 21)} = 8.476$, $P = 0.002$]. Data are presented as means ± SEM ($n = 8$ per group) and analyzed by one-way ANOVA followed by Tukey's *post hoc* test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. CD, cognitive dysfunction; NCD, non-cognitive dysfunction; N.S., not significant.

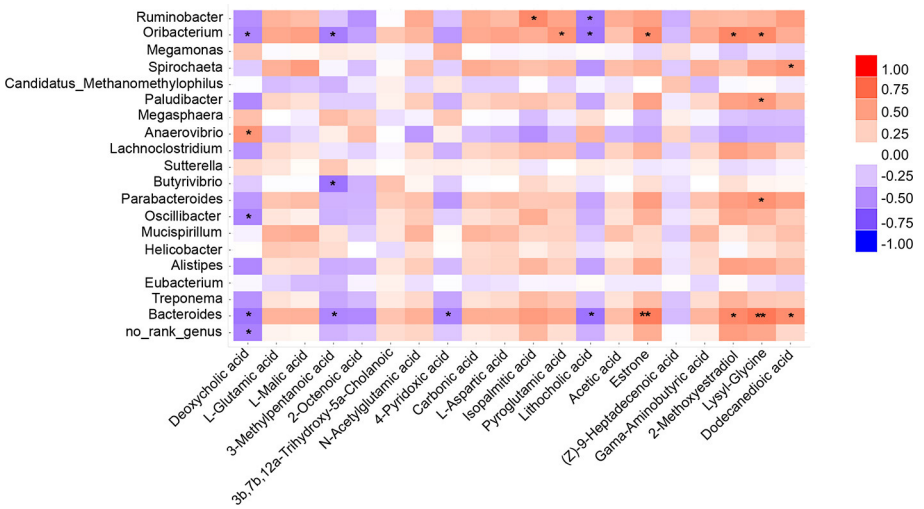

FIGURE 5 | Correlations between plasma metabolite and gut microbiota. Heatmap indicated that some microbes were positively correlated with plasma metabolites and some were negatively correlated with metabolites. $^*P < 0.05$, $^{**}P < 0.01$.

or indirectly by eCB, including AEA, 2-arachidonylglycerol (2-AG), N-acylserotonins, and some N-acylethanolamines. Results showed that the mRNA expression of these receptors, with the exception of *ppara*, was reduced in antibiotic-treated mice. However, only *cnr1*, *cnr2*, and *htr1a* mRNA levels were rescued after receiving bacterial transplantation from the Sham group mice (**Figures 7A–G**). Therefore, it is likely that the eCB signaling throughout the body serves as a mediator in the cross talk between intestinal bacteria and behavioral performance in the development of chronic pain and CD.

DISCUSSION

Evidence suggests that CD is one of the most common comorbidities of chronic pain, especially in CNP (Chou et al., 2007; Landro et al., 2013). In this study, SNI mice were clustered into CD (54.8%, 17/31) and NCD (45.2%, 14/31) phenotypes using the hierarchical cluster analysis according to the MWMT performance indices, while mice in CD and NCD groups suffered identical nociception. Compared to the NCD mice, the CD mice exhibited poor learning and memory ability, suggesting that the hierarchical cluster analysis is an effective approach to discriminate those without CD in mice after nerve injury. Consistent with our previous report (Yang et al., 2019), the 16S rRNA diversity analysis showed a consistent decrease in the richness and evenness of gut bacteria in SNI mice when compared with the Sham group, suggesting that alteration in gut microbiota was mainly influenced by chronic pain compared to cognitive disorders. However, the PCA results of plasma metabolites could separate the CD group from the NCD group. Collectively, it is likely that plasma metabolites play a pivotal role in these behavioral abnormalities of cognitive disorder after SNI when the structural dysbiosis of gut microbiota occurred.

Previous studies have demonstrated that some bacterial members from the four phyla (*Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*), such as *Bacteroides*, *Lachnospiraceae*, and *Enterobacteriaceae*, modulate chronic pain and cognitive disorders (Gareau, 2016; Proctor et al., 2017; Li et al., 2020; Chen et al., 2021). In this study, we observed that the relative abundance of *Actinobacteria* was higher in CD than in Sham and NCD mice. Moreover, LDA results indicated that *Actinobacteria* was one of the most important features classifying CD phenotypes in SNI mice. It has been reported that *Actinobacteria* was increased in interstitial cystitis/bladder pain syndrome but decreased in fibromyalgia (Braundmeier-Fleming et al., 2016; Minerbi et al., 2019). In addition, the relative abundance of *Actinobacteria* was increased in patients with Alzheimer's disease (AD) but decreased in triple-transgenic (3xTg) AD mouse model (Zhuang et al., 2018; Bello-Medina et al., 2021). This inconsistency may be attributed to the fact that both potential pathogens and probiotics were contained in the *Actinobacteria* phylum. Collectively, these results suggest that abnormal outgrowth of *Actinobacteria* might contribute to the pathogenesis of CNP and its associated cognitive deficits.

Among the top 10 most significantly differential genera, most of them ($n = 8$) showed consistent changes in the NCD and

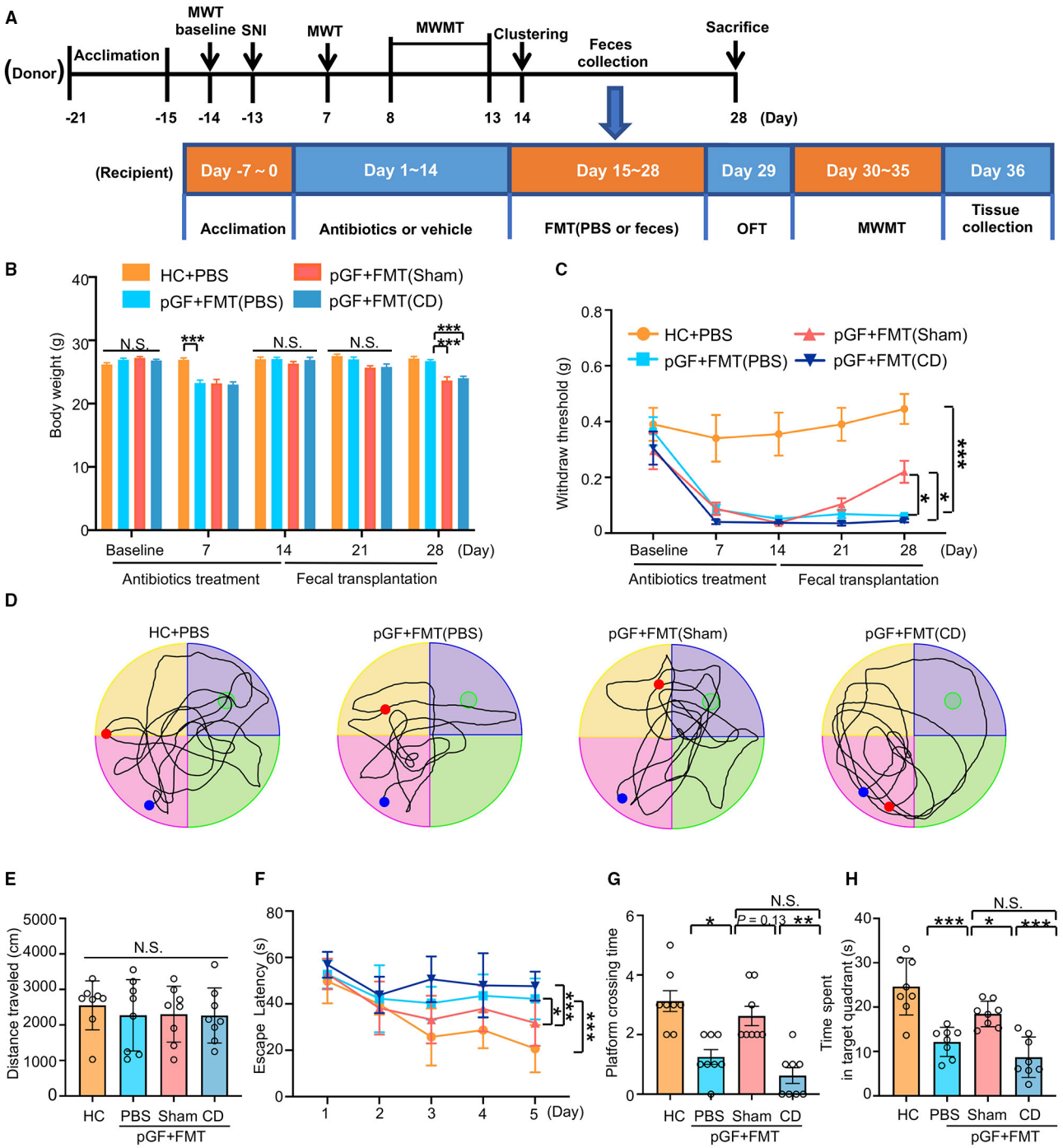

FIGURE 6 | Fecal microbiota transplantation (FMT) from mice of Sham reversed nociception and cognitive impairment in pseudo-germ-free (pGF) mice. **(A)** Experiment schedule of the fecal microbiota donor and receptor mice. To ensure the vitality of the intestinal flora, 1 g fresh feces were collected from the donor mice and then immediately dissolved in 10 ml phosphate-buffered saline (PBS) to prepare suspension for later FMT. For the donor mice, 10-month-old C57 mice were acclimated to the environment from day 21 to day 15 and performed SNI on day 13. MWMT was carried out from day 8 to day 13. After clustering into SNI with CD or NCD group, feces were collected before the FMT from day 15 to day 28. Donor mice were sacrificed after feces collection on day 28. For the receptor mice, 2-month-old C57 mice were treated with broad-spectrum antibiotics in drinking water for 14 consecutive days (day 1 to day 14). Subsequently, mice were treated with 0.2 ml PBS or fecal microbiota suspension obtained from Sham and CD group mice by oral gavage once a day for 14 consecutive days (day 15 to day 28). MWT and body weight were measured on day 1 (baseline), 7, 14, 21, and 28 after SNI. The collection of tissues was performed after MWMT on day 35 after SNI. **(B)** Body weight [two-way RM ANOVA; Time: $F_{(4, 140)} = 41.8$, $P < 0.001$; Group: $F_{(3, 140)} = 24.76$, $P < 0.001$; Interaction: $F_{(12, 140)} = 8.544$, $P < 0.001$]. **(C)** MWT [two-way RM

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FIGURE 6 | ANOVA; Time: $F_{(4,140)} = 17.19$, $P < 0.0001$; Group: $F_{(3, 140)} = 48.48$, $P < 0.0001$; Interaction: $F_{(12, 140)} = 2.487$, $P = 0.006$]. **(D)** The graphs of the representative traces of control, pGF mice treated with PBS, or gut microbiota from the Sham or CD group mice in the probe test. **(E)** OFT [one-way ANOVA; $F_{(3, 28)} = 0.2$, $P = 0.88$]. **(F)** MWMT [two-way RM ANOVA; Time: $F_{(4, 140)} = 16.55$, $P < 0.001$; Group: $F_{(3, 140)} = 20.45$, $P < 0.001$; Interaction: $F_{(12, 140)} = 2.152$, $P = 0.017$]. **(G)** Platform crossing times [Kruskal–Wallis non-parametric test; $H_{(3, 28)} = 21.1$, $P = 0.0001$]. **(H)** Time spent in target quadrant [one-way ANOVA; $F_{(3, 28)} = 19.59$, $P < 0.0001$]. Data are presented as means ± SEM ($n = 8$ per group) and analyzed by one-way ANOVA or two-way ANOVA followed by Tukey's *post hoc* test or Kruskal–Wallis non-parametric test followed by Dunn's *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. CD, cognitive dysfunction; FMT, fecal microbiota transplantation; HC, healthy control; MWMT, Morris water maze test; MWT, mechanical withdraw threshold; NCD, non-cognitive dysfunction; N.S., not significant; OFT, open field test; pGF, pseudo-germ-free mice; SNI, spared nerve injury.

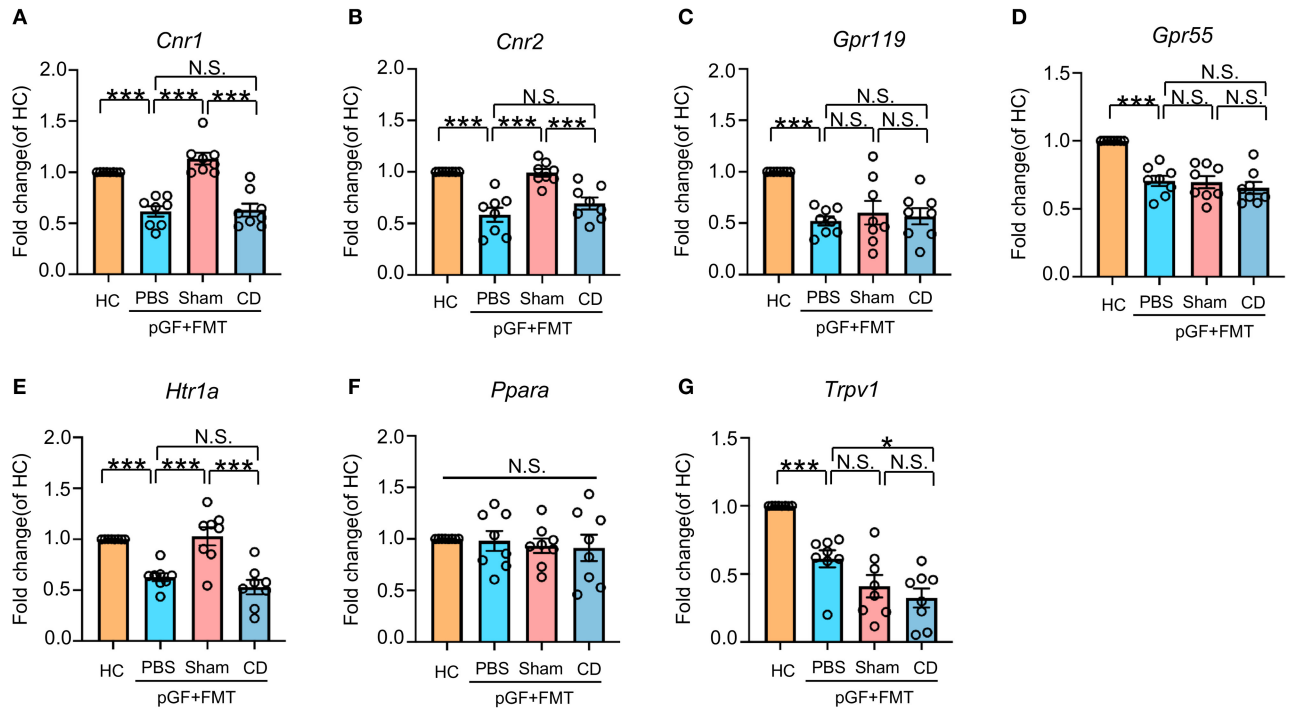

FIGURE 7 | Effects of fecal microbiota transplantation from mice in Sham and CD groups on endocannabinoid (eCB) receptors in pGF mice. **(A)** *Cnr1* [one-way ANOVA; $F_{(2, 21)} = 28.23$, $P < 0.001$]. **(B)** *Cnr2* [one-way ANOVA; $F_{(2, 21)} = 18.87$, $P < 0.001$]. **(C)** *Gpr119* [one-way ANOVA; $F_{(2, 21)} = 9.10$, $P < 0.001$]. **(D)** *Gpr55* [one-way ANOVA; $F_{(2, 21)} = 19.09$, $P < 0.001$]. **(E)** *Htr1a* [one-way ANOVA; $F_{(2, 21)} = 17.74$, $P < 0.001$]. **(F)** *Ppara* [one-way ANOVA; $F_{(2, 21)} = 0.21$, $P > 0.05$]. **(G)** *Trpv1* [one-way ANOVA; $F_{(2, 21)} = 23.48$, $P < 0.001$]. Data are presented as means ± SEM ($n = 8$ per group) and analyzed by one-way ANOVA followed by Tukey's *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. CD, cognitive dysfunction; FMT, fecal microbiota transplantation; HC, healthy control; N.S., not significant; pGF, pseudo-germ-free mice.

CD phenotypes, suggesting their important roles in CNP but not in cognitive deficits. Only *Proteus* (genus of *Proteobacteria*) and *Bifidobacterium* (genus of *Actinobacteria*) were associated with CNP-associated cognitive deficits. *Proteus* belongs to the *Enterobacteriaceae* family, which is a hallmark of dysbiosis in the gut (Rivera-Chavez et al., 2017). Notably, species in the *Proteus* are potentially pathogenic bacteria because they may exert pro-inflammatory effects in a host by producing lipopolysaccharide and flagellin proteins (Hamilton et al., 2018). Moreover, the LDA identified that *Proteus* was another gut flora feature that can be used to classify CD phenotype. Therefore, the enrichment of *Proteus* might have contributed to CNP-associated cognitive deficits. Mounting studies have revealed the protective roles of *Bifidobacterium* in various diseases *via* modulating the immune response, glycan metabolism of host, and microbiota-derived metabolites (e.g., short-chain fatty acids) (Turroni et al., 2016; Ruiz et al., 2017). Therefore, the strains of *Bifidobacterium* are widely used as probiotics. These benefits were also observed in studies involving chronic pain or cognitive impairment (Pokusaeva et al., 2017; El-Atawneh et al., 2019). *Bifidobacterium* could modulate visceral pain *via* the enzymatic decarboxylation of glutamate, which induced the production of γ-aminobutyric acid, an important neurotransmitter (Pokusaeva et al., 2017). Moreover, some strains showed therapeutic potential for age-related cognitive impairment, especially AD (El-Atawneh et al., 2019; Zhu et al., 2021), which indicated that *Bifidobacterium* might be beneficial for CNP-associated cognitive deficits. However, in this study, we observed a higher abundance of *Bifidobacterium* in the CD phenotype compared with the other two groups. This is consistent with a few studies that also found increased occurrences of *Bifidobacterium* in several neuropsychiatric disorders (Chung et al., 2019; Ling et al., 2020). In some cases, *Bifidobacterium* species were even regarded as potential pathogens (Pathak et al., 2014). These

results made it difficult to determine whether the increase of *Bifidobacterium* was a detrimental factor in SNI-associated cognitive deficits or just an adaptive/compensatory change in the dysbiosis. Therefore, further studies should be conducted to explore the different roles of *Bifidobacterium* species in CNP-associated cognitive deficits. Collectively, these findings suggest that an increased abundance of *Actinobacteria*, *Proteus*, and *Bifidobacterium* might be the important microbial signatures in CNP-associated cognitive deficits.

Non-targeted plasma metabolic profile was evaluated by LC-MS, and a total of 101 differential metabolites were obtained. The KEGG functional analysis showed that these metabolites were enriched in retrograde eCB signaling, autophagy, bile secretion, and lipids or amino acid biosynthesis and metabolism. Among them, alterations in the retrograde eCB signaling pathway were the most evident. The eCBs are lipid molecules and act as retrograde neurotransmitters that bind cannabinoid receptors and modulate synaptic efficacy (Mulder et al., 2011). The eCB signaling, including two components, namely, AEA and 2-AG, is involved in pain, emotion, stress, and cognition processes (Russo et al., 2018; Mecca et al., 2021; Rea et al., 2021). In this study, the plasma concentrations of AEA in SNI mice were significantly decreased, indicating its potential role in improving SNI-induced neuropathic pain. AEA modulates nociception in the peripheral nociceptors and CNS *via* cannabinoid receptors, TRPV1 channels, and, perhaps, GPR55 (Naz, 1987; Malek et al., 2014). Importantly, AEA is produced to exert anti-inflammatory and neuroprotective effects after nerve injury. However, various studies using different models consistently reported that increases, no changes, or decreases of AEA levels in the spinal cord might be present in chronic pain condition (Malek et al., 2014; Mecca et al., 2021), which were attributed to disparities in activities of receptors, synthesis, and degradation pathways. Various long-chain fatty acid amides, N-acylated amino acids/amines, and their congeners have been reported to exert eCB-like effects on pain and other neurological processes (Di Marzo, 2020). In this study, we established that several plasma fatty acids amides (e.g., N-stearoyl valine) and N-acylated amino acids (e.g., N-Acetylornithine) were significantly decreased in the plasma of SNI mice, consistent with AEA changes, partly due to the fact that this expanded eCB shared inactivating enzymes with AEA (Di Marzo, 2020). With regard to cognitive function, studies have reported that elevated AEA plasma levels in several cognitive disorders and that the pharmacological elevation of AEA may impair memory (Basavarajappa et al., 2014; Cristino et al., 2020). Even though its effects on cognition have not been conclusively determined, AEA elevation induces neuronal excitotoxicity by the long-term activation of the CB1 receptor (Cristino et al., 2020). Consistently, we found that the plasma levels of AEA and the expanded eCB were elevated in the CD phenotype, compared with the NCD phenotype. These findings indicate that, in chronic conditions, elevated AEA and other eCB may contribute to CNP-associated cognitive deficits although they have analgesic properties. Nonetheless, further study is needed to evaluate the role of receptors and metabolic routes of eCB in the comorbidity of CD with CNP.

Of the 61 different metabolites, most are mainly associated with the biosynthesis and metabolism of amino acids and lipids. Amino acids-derived metabolites include N-acyl-amino acids, dipeptides, branched amino acids, glutathione, and several tryptophan catabolites. Glutathione, an important antioxidant agent in the host, was significantly elevated in CD phenotypes, indicating the scavenging of excess reactive oxygen species when CNP-associated cognitive deficits occurred. The levels of four tryptophan catabolites, such as DHA-5-HT, Trp-P-1, indoleacetaldehyde, and Nb-palmitoyltryptamine, were found to be changed. DHA-5-HT is derived from the conjugation of a neurotransmitter (serotonin) and an omega-3 polyunsaturated fatty acid, docosahexaenoyl acid (DHA). Previous studies reported that DHA-5-HT exerted anti-inflammatory effects by inhibiting the interleukin (IL)-23-IL-17 signaling cascade (Poland et al., 2016; Wang et al., 2017). Moreover, it was shown to protect against glutamate-induced cytotoxicity *in vitro* by activating antioxidant enzymes in the CNS (Jin et al., 2014). In this study, DHA-5-HT levels were significantly suppressed in CD phenotypes, indicating the potential disorders of immunomodulation and oxidative stress in CNP-related cognitive deficits. The other three tryptophan catabolites are indole derivatives, which play important roles in the microbiota-gut-brain axis (Roager and Licht, 2018). Compared to DHA-5-HT, their changes were mainly due to the SNI procedure and were less associated with cognitive impairment. Sphingolipids and the derivatives of glycerophospholipids, such as phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS), are significantly differential lipid metabolites. They are important constituents of cell membranes and play important roles in cell signaling transduction. In this study, we found that glycerophospholipid metabolism was dysregulated in CNP associated with cognitive impairment. Besides, the levels of many phytochemical metabolites, such as americanin B, apritone, licoricone, and 2,3-di-O-methylellagic acid, were decreased in CNP-associated cognitive deficits (N10–19 in **Supplementary Table 2**). Most of them are polyphenols, which may regulate oxidative stress by the activation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway (Qin and Hou, 2016). A few differential metabolites are also associated with glucose (D-glucose, D-galactose) as well as bile acid (allocholic acid) metabolism, suggesting the disturbed energy and bile acid metabolism in mice with cognitive impairment under CNP condition. Overall, it is likely that the lipid and amino acid metabolism disturbances might be associated with cognitive deficits in mice with neuropathic pain.

To investigate the role of gut microbiota composition in CNP-associated cognitive deficits, we constructed pGF mice using an antibiotic mix and FMT of the Sham group and CD phenotype into their gastrointestinal tracts. Diminished microbiota and FMT of CD phenotype models induced hyperalgesia and cognitive deficits. Moreover, FMT in the Sham group improved allodynia (at 1 week) and cognitive performance. Given that the eCB signaling pathway was the most significant one in the KEGG analyses, and its promising therapeutic effects on CNP have been widely validated (Starowicz and Finn, 2017; Guida et al., 2020),

we evaluated the expressions of several eCB receptors in PFC. PFC, a crucial region, is associated with pain processing and cognition, due to its close connection with other brain areas, such as the hippocampus, amygdala, and thalamus (Baliki et al., 2006; Ong et al., 2019). Diminished microbiota and FMT in the CD phenotype models suppressed the levels of almost all the eCB system-related receptors, except *ppara*. However, FMT in the Sham group only rescued the expressions of *cn1r*, *cn2r*, and *htr1a*, indicating that the interaction between gut microbiota and eCB system plays a pivotal role in the pathogenic processes in CNP and its associated cognitive deficits.

Recently, growing evidence suggests that eCB system is an important player during the cross talk between gut microbiota and host (Cani et al., 2014). Gut microbiota has been shown to produce eCBs and many bioactive lipids that structurally and functionally resemble eCBs (Cani et al., 2016; Sharkey and Wiley, 2016). Given that the eCB system plays an important role in energy metabolism and inflammation, it may act as a promising therapeutic target for many neurological diseases (Minichino et al., 2021). In this study, we observed that AEA and many expanded eCBs showed significant decreases in the blood when pain and its associated cognitive deficits occurred. Moreover, the changes of FMT in pGF mice demonstrated that gut microbiota could modulate the expressions of eCB receptors in the CNS. These results indicated that eCB system might act as an important mediator between microbiota and the neurological changes induced by CNP. Gut dysbiosis in CNP may cause the declines of eCB ligands and receptors in the brain, resulting in the disorders of eCB signaling transduction, which may aggravate neuroinflammation and the imbalance of brain energy metabolism, finally present as CD. This potential mechanism based on the microbiota-gut-brain axis should be further validated in CNP and its associated cognitive deficits.

CONCLUSIONS

Different compositions of gut microbiome and plasma metabolome were present in CNP-related cognitive impairment models. Peripheral nerve injury dysregulated gut microbiota composition and related metabolites, resulting in decreased cognitive performance. Furthermore, in CNP-associated cognitive impairment, the increased abundance of *Actinobacteria*, *Proteus*, and *Bifidobacterium* are the important microbial signatures, while metabolomic signatures might be dominated by disturbances in the eCB system, lipid, and amino acid metabolism.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the MetaboLights repository, accession number MTBLS3891, www.ebi.ac.uk/metabolights/MTBLS3891.

ETHICS STATEMENT

The animal study was reviewed and approved by Experimental Animal Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology.

AUTHOR CONTRIBUTIONS

DH, ShaL, and AL designed this study and wrote the manuscript. DH, XW, YW, ZX, and YZ performed the experiments. ShiL and JZ analyzed the data. AL revised the manuscript. AL and JZ funded this study. All authors contributed to this study and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2021.806700/full#supplementary-material

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Mechanism of Mongolian Medicine *Eerdun Wurile* in Improving Postoperative Cognitive Dysfunction Through Activation of the PI3K Signaling Pathway

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Objective: To study the effect of *Eerdun Wurile (EW)*, a traditional Mongolian medicine, on the cognitive function of rats by activating the IRS-PI3K-AKT-GLUT4 pathway in an animal model of postoperative cognitive dysfunction (POCD).

Methods: Fifty clean-grade adults Sprague Dawley (SD) male rats were assigned to one of five groups: (1) a control group with no anesthesia (Group C), (2) a POCD model group with anesthesia only (Group P), (3) POCD group with low-dose EW treated (Group L), (4) a POCD group with high-dose EW treated (Group H), and (5) a POCD model group with dexmedetomidine treated (Group D) for positive control. The study started 7 days after all rats had acclimated to housing. Rats were trained in the Morris Water Maze navigation 5 days before surgery. All rats underwent the same maze for navigation and spatial exploration experiments on the preoperative day 1 and postoperative days 1, 3, 5, and their learning and memory abilities were assessed. At the end of the water maze experiment, rats were sacrificed to obtain hippocampal tissue. The mRNA levels of IRS-2, PI3K, AKT, and GLUT4 were measured in the hippocampus by real-time PCR, and the expression of IRS-2, PI3K, AKT, and GLUT4 protein in the hippocampus was determined by Western blotting to investigate the potential mechanisms at the molecular level.

Results: Compared to control Group C, Group P, L, H, and D showed prolonged escape latency ($P < 0.05$) and decreased number of times to cross the platform ($P < 0.05$) at 1, 3 and 5 days after surgery. Compared to Group P, Group L, H, and D showed a decrease in escape latency with an increased number of crossing the platform at all-time points after surgery ($P < 0.05$). Within individual P, L, H, and D groups, escape latencies decreased ($P < 0.05$) and the number of times that the platform was crossed increased ($P < 0.05$) between postoperative days 3 and 5 compared to postoperative 1 day. Compared to Group C, the mRNA expression of IRS-2, PI3K, AKT and GLUT4 in the hippocampus of P, L, H, and D groups were decreased ($P < 0.05$). Compared to Group P, IRS-2, PI3K, AKT, and GLUT4 in the hippocampus of L, H, and D groups were increased ($P < 0.05$). Compared with Group D, the expression levels of IRS-2 and AKT in both L and H groups were higher. The expression level of PI3K in Group L

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was also higher ($P < 0.05$) vs Group D. The expression of AKT mRNA in Group H was higher than in Group L ($P < 0.05$). Compared to Group C, the p-IRS-2/IRS-2 ratio in the hippocampus of Group P was higher than that of Group C ($P < 0.05$). Compared to Group P, the ratios of p-IRS-2/IRS-2 in Group L, Group H, and Group D were lower, and the ratios of the p-PI3K/PI3K, p-AKT/AKT, and p-GLUT4/GLUT4 were higher ($P < 0.05$).

Conclusion: Administration of EW showed the effect on the signaling pathway in rats with POCD. The therapeutic effect was better in the low-dose group. This could be related to the insulin downstream signal molecule PI3K and the IRS-PI3K-AKT-GLUT4 signaling pathway.

Keywords: postoperative cognitive dysfunction, hippocampal formation, signaling pathway, Mongolian medicine *Eerdun Wurile*, PI3K-AKT pathway

INTRODUCTION

Postoperative cognitive dysfunction (POCD) is a global public health issue with an aging population that needs to be urgently solved. A total of 9–54% of adults over 65 years of age have been reported to develop POCD within 1 week after surgery (Androsova et al., 2015). As the population ages in China and the number of critically ill patients undergoing surgeries increases, the yearly incidence of POCD may increase. This will have a negative impact on the quality of life in older patients (Du and Weidong, 2019) and on social resources. The Mongolian medicine Eerdun Wurile (EW) produces apparent therapeutic effects on memory deterioration, cognitive decline, nerve palsy, and cerebral spinal cord damage. EW has been used to treat Sa disease (a cerebrovascular disease characterized by sudden dizziness, hemiplegia and unclear speech) along with Zachong-13 by Shan and Xia (2016), and has presented a satisfactory effect on symptoms of retardation of expression, memory deterioration, dizziness, and drowsiness. Furthermore, EW can significantly improve the neurobehavioral function of rats with a middle artery obstruction/reperfusion injury, narrow cerebral infarction area, and alleviate cerebral edema (Lian et al., 2014). Meanwhile, it also inhibits cell necrosis in the brain prefrontal cortex (Lian et al., 2016), and suppresses nerve cell apoptosis caused by ischemia and hypoxia injury in rat brain tissues (Chun et al., 2013). Preliminary results of this project have demonstrated abnormal glucose metabolism in the brain of adult POCD rats. As the insulin signaling pathway genes IRS-2, PI3K, AKT, and GLUT4, which are intimately related to glucose metabolism, have been down-regulated, this signaling pathway is considered involved in the POCD process (Du et al., 2019). Therefore, we hypothesized that EW would have a therapeutic effect on POCD via its potential regulatory mechanism on abnormal glucose metabolism.

MATERIALS AND METHODS

Animal Grouping

A total of 50 clean-grade adult male Sprague Dawley (SD) rats aged 10–12 months, weighing approximately 500 g, were provided by Beijing Xinglong Experimental Animals Co., Ltd. The rats were divided into five groups ($n = 10$) using a random number table method, including a normal control group (Group C) without anesthesia, a POCD model group without any treatments (Group P), a low-dose EW group (Group L), a high dose EW group (Group H), and a dexmedetomidine group (Group D) as a treatment control group. All the rats were raised in a clean environment with 12 h light and dark alternatives. Food and water were available at will. The breeding room temperature was set at 20–24°C. The experiment was started after 7 days of adaptive feeding. The specific grouping method was as follows:

1) Group C: rats were not given special treatment, except an equal amount of distilled water was administered by gavage.
2) Group P: an equal amount of distilled water was administered before surgery by gavage. One hour before surgery, the rats were intraperitoneally injected with 100 μg/kg lipopolysaccharide (LPS), anesthetized using 10% chloral hydrate (0.3 mL/100 g), and a left nephrectomy was performed.
3) Group L: low-dose EW (0.63 g/kg) was administered by continuous intragastric administration 3 days before surgery. One hour before surgery, the rats were intraperitoneally injected with 100 μg/kg LPS, anesthetized using 10% chloral hydrate (0.3 mL/100 g), and a left nephrectomy was performed. Low-dose EW was administered by gavage 2 h after surgery, and an equal volume was administered by gavage daily until the 5th day after surgery.
4) Group H: high dose EW (1.26 g/kg) was administered by continuous intragastric administration 3 days before surgery. One hour before surgery, rats were peritoneally injected with 100 μg/kg LPS, anesthetized using 10% chloral hydrate (0.3 mL/100 g), and a left nephrectomy was performed. High dose EW was administered by gavage 2 h after surgery, and an equal volume was administered by gavage daily until the 5th day after surgery.
5) Group D: rats were injected with 100 μg/kg LPS (IP, 1 h before the surgery) and 25 μg/kg dexmedetomidine hydrochloride (IP, 15 min before surgery) and then anesthetized using 10% chloral hydrate (0.3 mL/100 g), followed by left nephrectomy. The oral dose of EW for an adult is 13–15 granules once or twice a day, 2 g/10 granules, approximately 3 g/day.

We used this weighted-based dosing for rats. Rats were administered intragastrically at a dose per kilogram of body weight (low-dose administration: 0.63 g/kg and high dose 1.26 g/kg).

Behavioral Experiments

The Morris Water Maze included a cylindrical water tank as a hidden platform for animals to inhabit. The tank was divided into four quadrants: the first, second, third, and fourth quadrants. Each had a fixed place for the experimental animal and a heater to maintain the water temperature at around 24°C. The hidden habitat platform was 1 cm below the water level and 10 cm in diameter, placed in a certain quadrant for laboratory animals to find. A video analyzer was used to record the tracks, swimming speed, escape latency, and the number of crossings of the platforms of the animals in the water maze. All experimental rats were trained in positioning and navigation 5 days before surgery. Meanwhile, the video analysis system analyzed the running track and recorded relevant data, namely escape latency. All rats were placed in a randomly selected quadrant facing the wall 1 day before surgery, then 1, 2, 3, and 5 days after surgery (POD 1, POD 3, and POD 5). The swimming speed, the number of crossings in each quadrant, and the escape latency were recorded. Subsequently, the hidden platform was removed, a quadrant was randomly selected again, and the rats were placed in a water maze tank facing the wall for spatial exploration tests. The number of platform crossings within 60 s was recorded (Chong et al., 2016).

Modeling and Material Selection

Except for Group C, all experimental rats of Group P, L, H, and D fasted for 12 h before surgery and intraperitoneally injected LPS 100 μg/kg 1 h before surgery. Additionally, five min before surgery, the animals were intraperitoneally injected with 0.3 mL/100 g 10% chloral hydrate mixture as anesthesia. When the rats lost their reflex, the fur under the left costal margin was shaved to prepare the skin. The animal was fixed on the operating bench in a right lateral position. Routine iodophor disinfection was performed twice before surgery, and sterile towels were spread. A longitudinal incision of 2–3 cm in length was made along the midaxillary line under the left costal margin. The abdominal cavity was dissected to fully expose the renal pedicle. After ligation of the ureter, renal artery, and renal vein at a distance of 0.5 cm from the renal hilum, the left kidney was removed. When the properpro reflex was regained, the rats were delivered to the breeding house to continue feeding. Combined with water maze tests and related laboratory examinations, the occurrence of POCD after surgery was determined. On POD 5, when all the rats received and completed behavioral experiments, they were anesthetized with 10% chloral hydrate, and the decapitated and hippocampal tissues were subsequently removed. Real-time fluorescence quantitative PCR assay was used to determine the mRNA expression of the relevant factors, and the operating procedures followed the instructions of the kit (Tiangen Biotech Co., Ltd.). The Ct value of each sample was obtained using the real-time quantitative PCR technique. The primer sequences are shown in **Table 1**.

TABLE 1 | Primer sequences.

Gene	Forward primers (5′-3′)	Reverse primers (5′-3′)
IRS-2	TGAGAGCGAGAAGAAGTGGAAG	CTTGGTGTAGAGGGCGATCAG
PI3K	GTCGTTGATAGACCACCGCTTCC	TGCCCTGTTCCTCTGCCTTCC
AKT	CAAGCACCGTGTGACCATGA	TCAGTAAGCGTGTGGGCAAC
GLUT4	CCAGTATGTTGCGGATGCTATG	GAAGGTGAAGATGAAGAAGCCAAG
β-actin	GTGCTATGTTGCTCTAGACTTCG	ATGCCACAGGATTCCATACC

Western blot detection was used to determine the protein expression levels of the relevant factors. The tissues were lysed with RIPA lysis buffer for protein extraction. After the RIPA lysate was dissolved, PMSF was added at the volume ratio of RIPA:PMSF = 100:1, and placed on ice for subsequent use. After lysing the tissue lysate in an ice bath for 30 min, centrifugation was performed at 12,000 rpm 4°C for 15 min and stored at −20°C or −80°C for later use after the supernatant. The BCA method was used to determine the protein concentration. The samples were added with a 5× loading buffer and boiled for eight min. Proteins were separated by SDS-PAGE gel electrophoresis, transferred to the NC membrane using a wet transfer method, and blocked with 5% skim milk at room temperature for 1 h. The primary antibody was used with the primary antibody diluent according to the described ratios: IRS-2 (1:1 000, Cell Signaling Technology, United States), PI3K (1:1 000, Cell Signaling Technology, United States), AKT (1:1 000, Cell Signaling Technology, United States), and GLUT4 (1:1 000, Proteintech, United States). The primary antibody was supplemented and incubated overnight at 4°C. The primary antibody was then collected, washed twice using an appropriate amount of 1× TBST, 8 min each time, and washed twice again with an appropriate amount of TBS, 5 min each time. After secondary antibody incubation and washing, TBS was removed, and the NC membrane was quickly transferred to an EP tube with secondary antibody diluted at 1:1 000 and incubated at room temperature for 1 h. The NC membrane was scanned using an infrared fluorescence analyzer, and the supporting software was utilized to analyze the gray value of the protein bands. The gray value ratio of the target band to the internal reference band was used as the expression level of the target protein in each group. Each sample was analyzed three times, and the average and standard deviation were calculated.

Statistical Methods

SPSS 22.0 software was used for statistical analysis. The measurement data were tested for normality and homogeneity of the variance, and the normal distribution was expressed as mean ± SD ($x \pm s$). One-way analysis of variance was used for comparison among groups, and analysis of variance with repeated measurement design was used to compare different time points within groups. $P < 0.05$ was considered statistically significant.

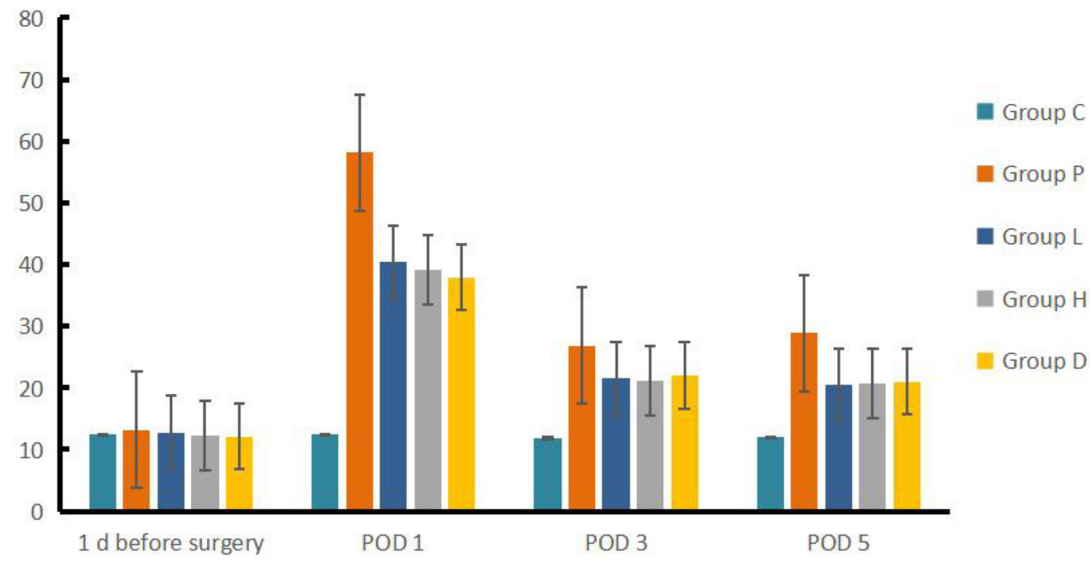

FIGURE 1 | Comparison of escape latency among the five groups.

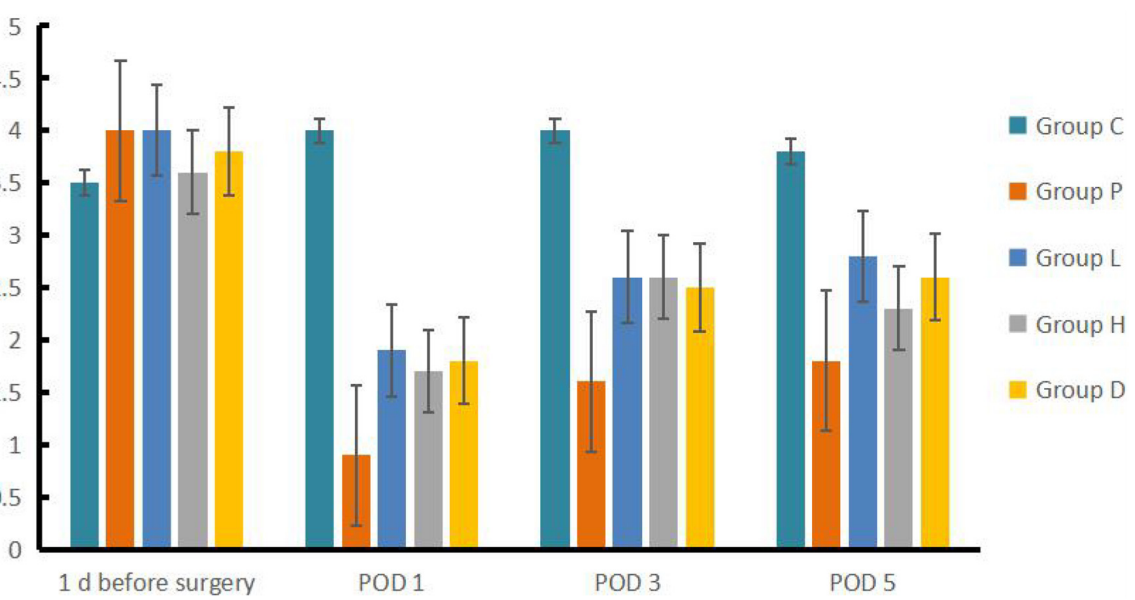

FIGURE 2 | Comparison of the number of crossing platforms among the five groups.

TABLE 2 | Comparison of mRNA levels of IRS-2, PI3K, AKT, and GLUT4 genes in hippocampal tissues the five groups (n = 10, $x \pm s$).

	Group C	Group P	Group L	Group H	Group D
IRS-2	1.028 ± 0.291	0.347 ± 0.0716[a]	0.665 ± 0.078abd	0.706 ± 0.109[ab]	0.489 ± 0.06[abc]
PI3K	1.001 ± 0.049	0.342 ± 0.086[a]	0.729 ± 0.109abcd	0.505 ± 0.114[ab]	0.498 ± 0.084[ab]
AKT	1.0 ± 0.035	0.31 ± 0.063[a]	0.664 ± 0.175abcd	0.803 ± 0.168[ab]	0.491 ± 0.083[abc]
GLUT4	1.002 ± 0.077	0.323 ± 0.06[a]	0.699 ± 0.094ab	0.663 ± 0.153[ab]	0.688 ± 0.073[ab]

A represented $P < 0.05$ compared to Group C; b represented $p < 0.05$ compared to Group P. c represented $P < 0.05$ compared to Group H; d represented $P < 0.05$ compared to Group D.

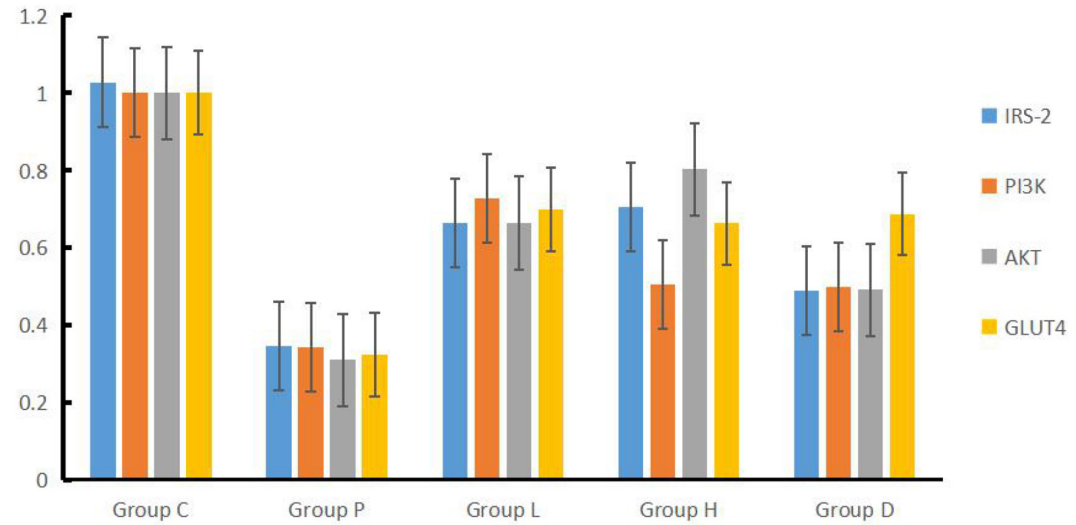

FIGURE 3 | Concentrations of IRS-2, PI3K, AKT, and GLUT4 factors in hippocampal tissues among the five groups.

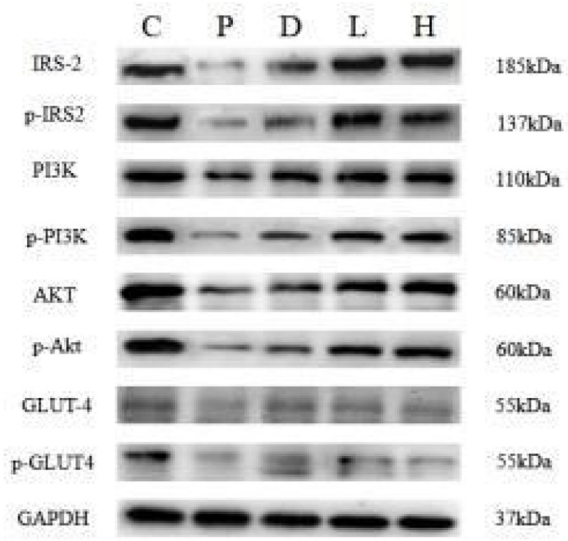

FIGURE 4 | Comparison of expressions of each factor in hippocampal tissues among five groups.

RESULTS

Compared to Group C, Group P, L, H, and D had a prolonged escape latency at POD 1, 3 and 5 ($P < 0.05$), and the number of crossing platforms decreased ($P < 0.05$). Compared to Group P, Group H, L, and D showed reduced escape latency but an increased number of platform crossings at each time point after surgery ($P < 0.05$). Compared to Group P, Group L, H, and D escape latency was lower at POD 3 and POD 5 than on POD 1 ($P < 0.05$), whereas the number of crossing platforms increased ($P < 0.05$) (see **Figures 1**, **2**).

Compared to Group C, the expressions of IRS-2, PI3K, AKT, and GLUT4 genes mRNA in the hippocampal tissues of Group P, H, L, and D decreased ($P < 0.05$). On the other hand, and compared with Group P, the mRNA expressions of the four genes described in Group H, L, and D increased ($P < 0.05$). In addition, compared with Group D, IRS-2 and AKT mRNA expression levels in Group L were higher, PI3K mRNA expression levels in Group L were higher than in Group D ($P < 0.05$), and mRNA expression of AKT in Group H was higher than that in Group L ($P < 0.05$), see **Table 2** and **Figure 3**.

Compared to Group C, the p-IRS-2/IRS-2 ratio of the hippocampal tissues in Group P was higher than that of the control group, and the difference was statistically significant ($P < 0.05$). Meanwhile, compared to Group P, the P-IRS-2/IRS-2 ratio in Groups H, L, and D was low, while the p-PI3K/PI3K, p-AKT/AKT ratios p-GLUT4/GUT4 genes increased ($P < 0.05$), as shown in **Figure 4**.

DISCUSSION

Based on the results of the water maze, we found that the rats in Groups P, L, H, and D had statistically significantly longer escape latency in POD 1, 3, and 5, along with a reduction in the number of crossing platforms compared to both 1 day before surgery by their own groups and Group C at each studied time after surgery, indicating that the establishment of the model was successful. We did find that EW has effects on POCD in rat model insults from both exploration anesthesia and left nephrectomy in Group L and H, similar to the effect of Group D as the therapeutic control of dexmedetomidine, and better than the non-treatment Group P in both latency and the number of crossing platforms compared to both 1 day before surgery by their own groups and Group P at every studied time after surgery. However, the effects did not appear to be dosing dependent given the fact that the number of

crossing platforms in Group L was higher than that in Group H in POD 5. Furthermore, as a positive control group, D may have a different pathway than Group H and L, as the mRNA expressions of the four genes were lower than those of Group L and H.

The specific mechanism of POCD pathogenesis remains unclear and is probably multifactorial. Normal glucose metabolism is known to lead to a series of vascular and neurodegenerations that impair cognitive function. In the case of insulin-induced severe hypoglycemia, the brain glucose level rapidly descends, leading to cognitive dysfunction, namely seizure and coma (Nacca et al., 2018). Alternatively, hyperglycemia can damage cerebral vascular tissues by injuring blood vessels, altering metabolism and neuronal calcium homeostasis, and causing changes and inflammatory reactions. This can lead to cognitive dysfunction and problems with the processing and integration of information, leading to impaired cognitive response and processing capacity (Feinkohl et al., 2019). Additional studies have shown that intraperitoneal injection of LPS can cause severe central nervous system degeneration (Fidalgo et al., 2011). However, some recently published systematic reviews and meta-analyses have shown that metabolic disorders in patients, including elevated blood glucose, blood pressure, and obesity, are closely correlated with the appearance and development of POCD (Hovaguimian et al., 2018). As shown previously, PET/CT detected abnormal glucose metabolism in the brain of adult POCD rats, further confirming that POCD was related to the glucose metabolism disorder in the brain (Du et al., 2019). Furthermore, abnormal energy metabolism is the main pathological cause of the occurrence and development of neurocognitive dysfunction after surgery. Information on the selection of lipopolysaccharide dosage, surgical model, and dosage can be found in the literature (Wang, 2016).

The hippocampal tissue factors have shown a similar pattern indicating these factors would be highly linked to the data shown in the water maze results as one of the potential causal effects or mechanisms. The results of quantitative fluorescence PCR indicated that the mRNA expressions of the IRS-2, PI3K, AKT, and GLUT4 genes in the hippocampal tissues of Group P, L, H, and D decreased compared to Group C. Compared to Group P, all the expressions of the four described genes in Groups L, H, and D were elevated. The results revealed that the abnormality of insulin signaling pathway IRS-PI3K-AKT-GLUT4 at the genetic level might be the mechanism of POCD-induced cerebral glucose metabolism disorder caused by surgery combined with peripheral inflammation. Western blot findings were consistent with the expressions at the genetic level, which further verified that the insulin signaling pathway in the hippocampal tissues of POCD rats was disordered and glucose metabolism abnormal, consistent with the finding of the maze data.

The study has certain limitations. First, since chloral hydrate has no analgesic effect, anesthesia with it has the potential to interfere with the outcome of this experiment and is inhumane to experimental animals. Later, we will further standardize the use of anesthetics to establish a more regulated model to observe the effects of different anesthetics on the results of this study. Second, this experiment explains the changes in human body mechanisms from the perspective of animals, but there are still differences between animals and humans. There is still a long way to go for this study to be applied to clinical practice in the future. Third, we are uncertain whether EW acts directly or indirectly on this pathway to exert its effect, and further studies are needed in the future. Furthermore, in the escape study, all treated groups (except for Group C) had varying levels of latency. Except for the reason of POCD, it could be due to postoperative pain. In Groups H, D, and L, the medicine is given may have different levels of analgesic functions. However, studies on postoperative analgesia by EW are rarely reported.

This experiment is the first step taken by our research group on the impact of EW on POCD. There are many problems to be solved. For example, we are not sure why the effect of the low-dose group is better than that of the high-dose group, and what role does the concentration of EW play? Also, will anesthesia with chloral hydrate affect the results of this experiment? This needs to be confirmed by further research in the future.

Taken together, changes in this signaling pathway were observed in rats with POCD. And the low-dose group could be more useful in the treatment of POCD. The mechanism may be correlated with the downstream insulin signaling molecule PI3K and the IRS-PI3K-AKT-GLUT4 signaling pathway involved in its regulation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving animals were reviewed and approved by the Medical Ethics Committee of Inner Mongolia Medical University No. YKD202101074.

AUTHOR CONTRIBUTIONS

YD, JY, DC, and LC designed and supervised the whole experimental process. ES, HL, and ZL performed the experiments. ZL and LC provided technical assistance and statistical analysis and reviewed and edited the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.769759/full#supplementary-material

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Transcutaneous Electrical Nerve Stimulation in Rodent Models of Neuropathic Pain: A Meta-Analysis

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Transcutaneous electrical nerve stimulation (TENS) is a non-invasive therapeutic intervention that is typically used for many years to treat chronic pain in patients who are refractory to pain medications. However, evidence of the efficacy of TENS treatment for neuropathic pain is lacking in humans. To further understand the efficacy of TENS under various intervention conditions and illuminate the current circumstance and future research directions, we systematically reviewed animal studies investigating the efficacy of TENS in relieving pain in neuropathic pain rodent models. We searched the Cochrane Library, EMBASE, MEDLINE (via PubMed), and Web of Science and identified 11 studies. Two meta-analyses were performed. The first meta-analysis showed that a single TENS treatment was capable of temporarily ameliorating neuropathic pain when compared to control groups with a significant effect (standardized mean difference: 1.54; 95% CI: 0.65, 2.42; $p = 0.0007$; $I^2 = 58\%$). Significant temporary alleviation in neuropathic pain intensity was also observed in the meta-analysis of repetitive TENS (standardized mean difference: 0.85; 95% CI: 0.31, 1.40; $p = 0.002$; $I^2 = 75\%$). Subgroup analysis showed no effect of the timing of the application of TENS (test for subgroup difference, $p = 0.47$). Leave-one-out sensitivity analyses suggested that no single study had an outsized effect on the pooled estimates, which may partly prove the robustness of these findings. Other stratified analyses were prevented by the insufficient number of included studies. Overall, current data suggest that TENS might be a promising therapy to ameliorate neuropathic pain. However, the high risk of bias in the included studies suggests that cautions must be considered when interpreting these findings and it is not reasonable to directly generalize the results obtained from animal studies to clinical practice. Future studies should pay more attention to improving the quality of study design and reporting, thereby facilitating the understanding of mechanisms underlying TENS treatment, reducing more potentially unsuccessful clinical trials, and optimizing the efficacy of TENS for people with neuropathic pain.

Keywords: transcutaneous electrical nerve stimulation, neuropathic pain, animal studies, pain models, meta-analysis

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INTRODUCTION

Neuropathic pain, which is caused by an injury or disease of the somatosensory system, is characterized by spontaneous pain, hyperalgesia, and allodynia and can be classified as peripheral or central neuropathic pain according to the site of injury or disease (Treede et al., 2008). It is estimated that the prevalence of neuropathic pain is 6.9% to 10% (van Hecke et al., 2014). Neuropathic pain represents an important source of chronic pain and dysfunction and causes a significant burden on people and society (Jensen et al., 2011). Therefore, the management of neuropathic pain should be important to ease its negative impact on activities of daily living and quality of life (Andrew et al., 2014; Leadley et al., 2014).

The mainstay of interventions for neuropathic pain is primarily pharmacological (Dworkin et al., 2013); however, for the large number of patients who cannot benefit from pharmacological intervention or who experience unwanted side effects, improving the ability to effectively relieve neuropathic pain with a non-pharmacological intervention such as psychological or physical treatment is crucial (Somers and Clemente, 2009; Gibson et al., 2017). Transcutaneous electrical nerve stimulation (TENS) is a non-invasive, safe, easy to administer, portable, and inexpensive technique that delivers pulsed electrical stimulation, which can be modified regarding frequency, current intensity, and duration, *via* two or more skin electrodes to stimulate underlying nerves for pain control and has an advantage of allowing patients to control their pain autonomously (Pal et al., 2020). The antinociceptive effect of TENS may involve peripheral receptors (Santos et al., 2013), spinal (Melzack and Wall, 1965; Wall and Sweet, 1967), and supraspinal mechanisms (Kalra et al., 2001; DeSantana et al., 2008, 2009). And the application of TENS is based on the pain gate theory, which proposes that the stimulation of large diameter (A-β) afferent fibers may close the pain gate and alleviate the pain (Melzack and Wall, 1965; DeSantana et al., 2008). Besides, TENS treatment has been shown to relieve pain by reducing the sensitization of dorsal horn neurons (Sabino et al., 2008), elevating levels of gamma-aminobutyric acid and glycine (Maeda et al., 2007; Somers and Clemente, 2009), and inhibiting glial activation (Matsuo et al., 2014). The two most common types of TENS treatment are high-frequency (50 or 100 Hz and above), low-intensity TENS and low-frequency (10 Hz or less), high-intensity TENS (Hurlow et al., 2012; Gibson et al., 2017). However, the proof of the efficacy of TENS for neuropathic pain is limited and the TENS parameter that would best treat neuropathic pain remains unclear. A Cochrane Review of TENS for neuropathic pain reported that they cannot confidently state whether TENS is efficacy for neuropathic control due to the low-quality evidence obtained from a small number of studies included in the meta-analysis, and the lack of clinical studies prevented further subgroup analyses, resulting in the optimal pattern of TENS remaining unknown (Gibson et al., 2017). Furthermore, studies suggested that electrical stimulation exerted an antinociceptive effect in a specific time window (Kerns and Lucchinetti, 1992; Su et al., 2018), whereas there were various inconsistencies amongst previous studies with respect to the nociceptive effect according to the timing of intervention tested (Somers and Clemente, 1998; Su et al., 2018). In contrast to human trials, animal studies are more exploratory and enable the additional design of independent variables and the control of confounders. Previous animal studies have explored the effect of TENS on neuropathic pain, but results have not been consistent (Somers and Clemente, 2009; Lin et al., 2015; Su et al., 2018). It is worth noting that systematic reviews of preclinical studies have the potential to inform future clinical trials and thereby ease translational challenges (Harman et al., 2020). Therefore, a systematic review of animal studies exploring the efficacy of TNES for neuropathic pain is important and desirable. However, no meta-analysis has assessed the antinociceptive effect of TENS in alleviating neuropathic pain.

Based on the above background, we focused on a single TENS and repetitive TENS, with the primary purpose being to assess the efficacy of TENS in relieving pain in rodent models of neuropathic pain. The second purpose was to evaluate whether the efficacy of TENS is influenced by the TENS parameters and experimental design. The third purpose was to clarify the current circumstance and future research directions of TENS.

MATERIALS AND METHODS

The present meta-analysis followed the guidelines of Preferred Reporting Items for Systematic Review and Meta-analyses (Johnson et al., 2004) (**Supplementary Material 1**). The protocol for this study was available online (registration number: INPLASY2021110104). No ethical approval was needed as all information was extracted from studies published previously.

Search Strategy

Animal studies investigating the efficacy of TENS for neuropathic pain were identified by searching electronic databases, including Cochrane Library, EMBASE, MEDLINE (*via* PubMed), and Web of Science. Search terms included pain, transcutaneous electrical nerve stimulation, muridae, and keywords that were confirmed following multiple pre-searches (**Supplementary Material 2**).

Criteria for Considering Studies for This Meta-Analysis

The inclusion criteria for this meta-analysis were as follows: (1) Animal studies using rodent models of neuropathic pain induced by one of the following methods: chronic constriction injury (CCI), spared never injury (SNI), spinal cord injury (SCI), spinal nerve ligation (SNL), nerve crush injury (NCI), viral infection for postherpetic neuralgia, plexus ablation, chemotherapeutics, streptozotocin administration, or central lesions (Velzen et al., 2021); (2) Rodents in experiment groups received all standard models of TENS with unlimited frequency, intensity, duration, and timing of intervention; (3) Neuropathic pain-inducing rodents in the control group should receive sham TENS or blank treatment, except usual anesthesia; (4) Studies had to provide quantitative data on pain, irrespective of the type of pain, which can be measured by a mechanical threshold, thermal threshold,

or cold threshold. And pain can be expressed as an absolute value or a percentage.

Exclusive criteria were as follows: (1) studies using rodent models of inflammation pain, non-inflammation pain, or cancer pain, and those utilizing non-rodent models, humans, or *ex vivo* and *in vitro* preparations; (2) studies in which pulsed electrical stimulation was delivered percutaneously such as electroacupuncture (EA) and percutaneous electrical nerve stimulation (PENS) or in which rodents received vagus/trigeminus nerve stimulation or acupuncture points stimulation, including, but not limited to transcutaneous electrical acupoint stimulation (TEAS); (3) TENS was utilized in conjunction with another intervention; (4) studies not including an independent control group that did not receive active TENS; (5) Conference abstract, editorial, review, and non-English publications.

According to the above criteria, two reviewers independently read the titles and abstracts of the retrieved records and eliminated apparently irrelevant studies. Subsequently, the full text of the remaining studies was retrieved, and two investigators independently assessed the studies for final inclusions. In case of ambiguity, we contacted the authors to provide additional information *via* email. Discrepancies were resolved through discussion, or by consulting a third investigator.

Data Extraction and Quality Assessment

TENS and control data about paw withdrawal thresholds expressed as an absolute value or a percentage after TENS treatment at identical time points were extracted. When quantitative data were not explicitly reported in text and supplementary materials, we extracted the data from figures using Engauge Digitizer (Huang et al., 2021). The primary outcome mechanical threshold was used in the meta-analysis if reported and available (91% of included studies), otherwise thermal threshold or cold threshold was used as an alternative. First author information, year of publication, species, strain, age, sex, weight, sample size per group, modeling methods, the protocol of TENS, parameters of TENS, the timing of TENS, type of control intervention, anesthesia used during TENS treatment, outcome measurement methods, adverse events, and so on were also extracted. Two authors independently extracted data and then discussed or consulted a third reviewer to resolve discrepancies. The authors of included studies would not be contacted to provide missing data which has not been peer-reviewed.

Two investigators independently evaluated the risk of bias of included studies utilizing the SYRCLE's risk of bias tool for animal studies (Hooijmans et al., 2014b). High-bias risk, low-bias risk, and unclear bias risk were used to grade the included studies. We discussed, or consulted a third investigator to make final decisions.

Data Analysis

All meta-analyses and graphical displays were conducted using RevMan 5.3 (The Cochrane Collaboration, Copenhagen, Denmark). A random-effects model was used due to the exploratory nature of animal studies and the anticipated heterogeneity. If methods of outcome measurement or forms of data expression were different among the included studies, we calculated a standardized mean difference (SMD) to summarize effects from studies in this meta-analysis; otherwise, a mean difference (MD) was used. To ensure that the results had the same directional value, we multiplied one kind of outcome by -1 if the change direction to reflect the relief degree of neuropathic pain was different. To prevent double-counting sample sizes of control animals, we split the animal number of the control group in case of studies using a single control group and multiple experimental groups. We used I^2 to evaluate the heterogeneity. Where comparable data were available from at least three studies, we planned subgroup analysis in the following domains: frequency, the timing of intervention, intensity, electrode placement, species, method of modeling, the timing of outcome measurement, and anesthesia used during intervention procedures. We evaluated the robustness of the results using leave-one-out sensitivity analyses. For studies that could not be included in the meta-analysis, we performed a descriptive summary. The publication bias would be analyzed using a funnel plot in case of at least 10 studies were included in a certain subgroup; otherwise, we would not analyze the publication bias.

RESULTS

Results of the Search

Figure 1 shows the flow diagram for search strategy and study selection process. The literature search was conducted on November 12, 2021. We initially retrieved 1,296 potentially eligible records, of which 23 studies from Cochrane Library, 214 from EMBASE, 772 from MEDLINE, and 287 from Web of Science. We removed duplicates with 1,094 records were left for title-abstract screening, resulting in 1,051 records being discarded, mostly because of irrelevant research topics and ineligible treatment modalities such as EA. Forty-three records were remained to determine their eligibility by carefully full-text screening, followed by 32 records were excluded from this review for various reasons. As a result, a total of 11 studies were included and all of them were included in the quantitative synthesis (Somers and Clemente, 1998, 2003, 2006, 2009; Nam et al., 2001; Inoue et al., 2003; Vera-Portocarrero et al., 2013; Cho et al., 2014; Matsuo et al., 2014; Lin et al., 2015; Su et al., 2018).

Characteristics of Included Studies

Table 1 shows the characteristics of studies included in the meta-analysis. Sample sizes of the included studies ranged from 16 to 66. Regarding the species employed in the included studies, 91% (10/11) of studies (Somers and Clemente, 1998, 2003, 2006, 2009; Nam et al., 2001; Inoue et al., 2003; Vera-Portocarrero et al., 2013; Cho et al., 2014; Lin et al., 2015; Su et al., 2018) employed Sprague Dawley rats, and the remaining one study (Matsuo et al., 2014) used ICR/JCL mice. In terms of sex and weight, male animals were used in all of the included studies and the weight of the animals varied across the included studies. For the neuropathic pain models, CCI (6/11) was the most common method for neuropathic pain modeling, followed by

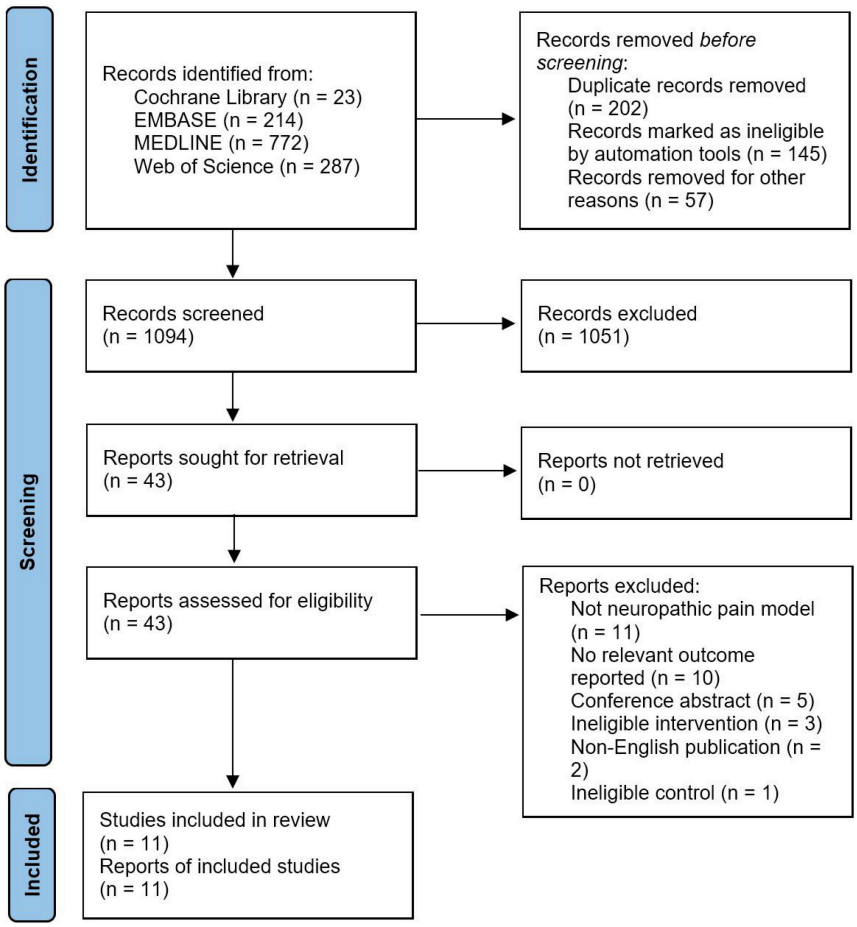

FIGURE 1 | PRISMA flow diagram for search strategy and study selection.

SNI (3/11), SNL (1/11), and NCI (1/11). With regard to the protocol of TENS, two studies (Nam et al., 2001; Cho et al., 2014) used a single session of TENS treatment and nine studies (Somers and Clemente, 1998, 2003, 2006, 2009; Inoue et al., 2003; Vera-Portocarrero et al., 2013; Matsuo et al., 2014; Lin et al., 2015; Su et al., 2018) employed repetitive TENS treatment, and of which one study (Vera-Portocarrero et al., 2013) provided the data about the effect of TENS following a single treatment. In addition, five studies (Somers and Clemente, 1998, 2006, 2009; Matsuo et al., 2014; Su et al., 2018) reported multiple separate comparisons (four in two studies, three in two studies, and two in one studies). As for the frequency of TENS, high-frequency was the most common one, which was used in seven studies (Somers and Clemente, 1998, 2003, 2006, 2009; Vera-Portocarrero et al., 2013; Cho et al., 2014; Matsuo et al., 2014; Lin et al., 2015), low-frequency was used in two studies (Nam et al., 2001; Inoue et al., 2003), and one study (Su et al., 2018) used both high-frequency and low-frequency TENS. Ipsilateral TENS was explicitly reported in seven studies (Somers and Clemente, 1998, 2003; Nam et al., 2001; Inoue et al., 2003; Cho et al., 2014; Matsuo et al., 2014; Lin et al., 2015), contralateral TNES was explicitly used in one study (Somers and Clemente, 2009), and one study compared the effect of ipsilateral and contralateral TENS (Somers and Clemente, 2006). However, two studies (Vera-Portocarrero et al., 2013; Su et al., 2018) did not explicitly which side of the body received TENS treatment. In terms of the intensity of TENS, all studies used sub-motor threshold, except one study (Nam et al., 2001) employed motor threshold and one study (Su et al., 2018) did not report on the intensity of TENS. Variations in the duration of TENS were observed, which ranged from 16.7 to 90 min. Regarding the control intervention, animals in seven studies (Somers and Clemente, 1998, 2003, 2006, 2009; Inoue et al., 2003; Lin et al., 2015; Su et al., 2018) received no TENS, and sham TENS was used in the remaining four studies (Nam et al., 2001; Vera-Portocarrero et al., 2013; Cho et al., 2014; Matsuo et al., 2014). Anesthesia was administrated during TENS treatment in all of the included studies, of which halothane (Somers and Clemente, 1998, 2003, 2006; Inoue et al., 2003) and isoflurane (Nam et al., 2001; Vera-Portocarrero et al., 2013; Lin et al., 2015; Su et al., 2018) were the most common anesthesia, followed by pentobarbital (Inoue et al., 2003). However, two studies (Cho et al., 2014; Matsuo et al., 2014) did not report on which type of anesthesia was used during TENS treatment.

TABLE 1 | Characteristics of studies included in the meta-analysis, $K = 11$.

Study	Animals					Modeling method	Parameters of TENS					Timing of intervention	Control intervention	Anesthesia used during intervention procedures
	Species	Strain	Age (wk)		Weight (g)		Frequency (Hz)	Intensity	Duration (min)	Electrical placement	Protocol			
Cho et al., 2014	Rat	Sprague Dawley	–	Male	200–250	SNI	100	Sub-motor threshold	20	Ipsilateral	Single TENS	4 weeks after modeling	Sham TENS	Brief anesthesia
Nam et al., 2001	Rat	Sprague Dawley	–	Male	150–200	SNL	2	Motor threshold	20	Ipsilateral	Single TENS	3 days after modeling	Sham TENS	2% enflurane-O_2 mixture
Vera-Portocarrero et al., 2013	Rat	Sprague Dawley	–	Male	250–300	SNI	100	90% of motor threshold	20	–	Daily for five consecutive days	7 days after modeling	Sham TENS	2–3% isoflurane
Inoue et al., 2003	Rat	Sprague Dawley	–	Male	250–280	CCI	1	The back of the rat extended vigorously and the head moved backward	16.7	Ipsilateral	Daily for five consecutive days	7th–11th day after modeling	None	Sodium pentobarbital 40 mg/kg i.p. or 2% halothane
Lin et al., 2015	Rat	Sprague Dawley	–	Male	200–250	CCI	100	80% of motor threshold	20	Ipsilateral	Daily for 13 consecutive days	One day after modeling	None	2% isoflurane
Matsuo et al., 2014	Mouse	ICR/JCL	9	Male	39.6	SNI	100	Sub-motor threshold	30	Ipsilateral	Daily for seven consecutive days	1 and 2 weeks after modeling	Sham TENS	Anesthesia
Somers and Clemente, 1998	Rat	Sprague Dawley	–	Male	150–165	CCI	100	80% of motor threshold	90 or 60	Ipsilateral	Daily for 14, 13, or 11 consecutive days	Immediately, 20–30 h, or 3 days after modeling	None	Halothane (4%, maintained at 0.2–0.5%)
Somers and Clemente, 2003	Rat	Sprague Dawley	–	Male	150–165	CCI	100	80% of motor threshold	90 on the first day and then 60	Ipsilateral	Daily for 12 consecutive days	Immediately after modeling	None	Halothane (4%, maintained at 0.2–0.5%)
Somers and Clemente, 2006	Rat	Sprague Dawley	–	Male	170–200	CCI	100	80% of motor threshold	60	Ipsilateral Contralateral	Daily for 12 consecutive days	Beginning on the day of modeling	None	Halothane (4%, maintained at 0.2–0.5%)
Somers and Clemente, 2009	Rat	Sprague Dawley	–	Male	150–175	CCI	100	80% of motor threshold	90 on the first day and then 60	Contralateral	Daily for 12 consecutive days	Beginning on the day of modeling	None	Halothane (4%, maintained at 0.2–0.5%)
Su et al., 2018	Rat	Sprague Dawley	–	Male	250–300	NCI	5 or 100	–	30	–	Daily for seven consecutive days	Immediately or 7 days after modeling	None	1% isoflurane

CCI, chronic constriction injury; i.p., intraperitoneal; SNI, spared nerve injury; SNL, spinal nerve ligation; TENS, transcutaneous electrical nerve stimulation; NCI, nerve crush injury.

As shown in **Table 2**, paw withdrawal threshold to a mechanical stimulus was used to measure neuropathic pain in 10 studies (Somers and Clemente, 1998, 2003, 2006, 2009; Nam et al., 2001; Vera-Portocarrero et al., 2013; Cho et al., 2014; Matsuo et al., 2014; Lin et al., 2015; Su et al., 2018), followed by thermal threshold was reported in eight studies (Somers and Clemente, 1998, 2003, 2006, 2009; Inoue et al., 2003; Cho et al., 2014; Matsuo et al., 2014; Su et al., 2018) and cold threshold in three studies (Nam et al., 2001; Vera-Portocarrero et al., 2013; Cho et al., 2014). With regard to the timing of outcome measurement, the short-term effects were reported in three studies using a single session of TENS (Nam et al., 2001; Vera-Portocarrero et al., 2013; Cho et al., 2014) and nine studies employing repetitive TENS (Somers and Clemente, 1998, 2003, 2006, 2009; Vera-Portocarrero et al., 2013; Matsuo et al., 2014; Lin et al., 2015), while two studies (Inoue et al., 2003; Su et al., 2018) explored the long-term effects of repetitive TENS (up to 14 and 28 days after TENS, respectively). None studies reported mortality and adverse events related to TENS treatment.

Quality Assessment

According to the SYRCLE's risk of bias tool for animal studies, the overall quality of existing literature was low due to the unclear risk of bias that existed in most of the studies (**Supplementary Material 3**). In terms of allocation sequence, we judged six out of the 11 included studies (Somers and Clemente, 1998, 2003, 2009; Inoue et al., 2003; Matsuo et al., 2014; Su et al., 2018) did not adequately describe the allocation sequence and the remaining five studies (Nam et al., 2001; Somers and Clemente, 2006; Vera-Portocarrero et al., 2013; Cho et al., 2014; Lin et al., 2015) did not report the random component in this process, and thereby we classified them as unclear risk for selection bias. Similarly, all the studies were rated as unclear risk for selection and detection biases because all the studies did not report or did not adequately report the method of allocation sequence concealment and whether the outcomes were measured randomly. Seven studies (Nam et al., 2001; Inoue et al., 2003; Vera-Portocarrero et al., 2013; Cho et al., 2014; Matsuo et al., 2014; Lin et al., 2015; Su et al., 2018) showed the intervention and control groups were comparable at baseline and thereby were rated as low risk of bias, while the other four studies (Somers and Clemente, 1998, 2003, 2006, 2009) did not report the similarity of groups and therefore were classified as unclear risk of bias. The majority of studies reported that animals were housed identically throughout the experiment and were rated as low risk of bias, except one study (Somers and Clemente, 1998) in which the housing conditions were omitted to report were classified as unclear risk of bias. In contrast, all of the included studies except one study (Vera-Portocarrero et al., 2013) were rated as unclear risk for performance bias due to the omitting of reporting whether TENS was performed in a blinded fashion. Only three studies (Nam et al., 2001; Lin et al., 2015; Su et al., 2018) did report the assessor was blinded, one study (Vera-Portocarrero et al., 2013) explicitly described the outcomes assessment did not conduct blindly, and the others did not mention this issue. Regarding attrition bias, six studies were classified as unclear risk of bias, of which three (Somers and Clemente, 1998, 2003; Su et al., 2018) did not report the total number of animals, two (Nam et al., 2001; Inoue et al., 2003) did not explain the reason why the animals dropped out, and the remaining one (Vera-Portocarrero et al., 2013) did not specify the number of animals per group. All of the included studies were rated as low risk for report bias, except one study (Su et al., 2018) that did not report the results of thermal threshold as planned was classified as high risk for report bias. Two studies were classified as unclear risk for other bias due to the lack of TENS intensity (Su et al., 2018) and the potential conflict of interest (Vera-Portocarrero et al., 2013). Four studies provided data related to the intra-rater reliability of the outcome measurement (Somers and Clemente, 1998, 2003, 2006, 2009).

Meta-Analysis 1: The Effect of a Single Session of Transcutaneous Electrical Nerve Stimulation on Neuropathic Pain

Three studies (Nam et al., 2001; Vera-Portocarrero et al., 2013; Cho et al., 2014) assessed the effect of a single session of TENS on neuropathic pain and all of them provided data that could be included in the meta-analysis for the neuropathic pain. Of these, the mechanical threshold data of one study (Cho et al., 2014) could not be extracted from the presented figure, thereby the thermal threshold result was used as an alternative.

Meta-analysis showed that a single session of TENS has a positive short-term effect in alleviating neuropathic pain relative to comparators (SMD: 1.54; 95% CI: 0.65, 2.42; $p = 0.0007$; $I^2 = 58\%$; **Figure 2**). The overall finding that a single session of TENS significantly alleviated neuropathic pain did not differ after omitting any single study of the included studies (**Supplementary Material 4A**). The number of included studies was too small to conduct reliable analyses of predefined subgroup and publication bias.

Meta-Analysis 2: The Effect of Repetitive Transcutaneous Electrical Nerve Stimulation on Neuropathic Pain

Nine studies (Somers and Clemente, 1998, 2003, 2006, 2009; Inoue et al., 2003; Vera-Portocarrero et al., 2013; Matsuo et al., 2014; Lin et al., 2015; Su et al., 2018) with 18 comparisons measured the efficacy of repetitive TENS for neuropathic pain. All of these studies provided available data of mechanical threshold, except one study measured neuropathic pain by the paw withdrawal threshold to a thermal stimulus and therefore the thermal threshold result was included as an alternative.

Overall, repetitive TENS was shown to have a positive effect in alleviating neuropathic pain. Repetitive TENS groups significantly ameliorated neuropathic pain relative to comparators (SMD: 0.85; 95% CI: 0.31, 1.40; $p = 0.002$; $I^2 = 75\%$; **Figure 3**).

The timing of the application of TENS relative to pain modeling was used to stratify the subgroups. However, the pooled SMDs did not differ significantly (test for subgroup difference, $p = 0.47$): the pooled SMD was 0.63 (95% CI: −0.20, 1.47; $p = 0.14$; $I^2 = 80\%$; **Figure 4**) for studies with TENS commencing on the day of modeling and 1.03 (95% CI: 0.33,

TABLE 2 | Characteristics of outcome evaluations, $K = 12$.

Times of TENS	Study	Mechanical threshold		Thermal threshold		Cold threshold		Timing of measurement	Adverse events
		Method	Relief of pain compared to control group	Method	Relief of pain compared to control group	Method	Relief of pain compared to control group		
Single session									
	Cho et al., 2014	von Frey Filaments	↑	Infrared generator	↑	Acetone	↑	Baseline and 30, 60, 90, 120, 180, and 240 h, and 1 day after TENS	–
	Nam et al., 2001	von Frey Filaments	↑	–	–	Ice	↔	30 min before, and at 30 min, 1, 2, 3, and 4 h after TENS	–
	Vera-Portocarrero et al., 2013*	von Frey Filaments	↔	–	–	Acetone	Unclear	Baseline and before and after TENS for five consecutive days	–
Multiple sessions									
	Inoue et al., 2003	–	–	Radiant heat	↔	–	–	Just before TENS, 7 days after modeling, and 1, 3, 7, and 14 days after the final TENS	–
	Vera-Portocarrero et al., 2013	von Frey Filaments	↔	–	–	Acetone	Unclear	Baseline and before and after electrical stimulation for five consecutive days	–
	Lin et al., 2015	von Frey Filaments	↑	–	–	–	–	Baseline and 3, 7, 11, and 14 days after modeling	–
	Matsuo et al., 2014	Analgesia-meter	Early↑ 1-week↔ 2-week↔	Radiant heat	Early↑ 1-week↔ 2-week↔	–	–	Before and every after modeling	–
	Somers and Clemente, 1998	Calibrate Semmes-Weinstein monofilaments	Immediately TENS ↔ 1-day TENS↔ 3-day TENS↑	Radiant heat	Immediately TENS↑ 1-day TENS↑ 3-day TENS↔	–	–	Baseline and then 2, 7, 12, and 14 days after modeling.	–
	Somers and Clemente, 2003	Calibrate Semmes-Weinstein monofilaments	↔	Radiant heat	↔	–	–	Baseline and 12 days after modeling	–
	Somers and Clemente, 2006	Calibrate Semmes-Weinstein monofilaments	High-frequency contralateral TENS↑ High-frequency ipsilateral TENS↔ Low-frequency contralateral TENS↔ Low-frequency ipsilateral TENS↔	Radiant heat	High-frequency contralateral TENS↔ High-frequency ipsilateral TENS↔ Low-frequency contralateral TENS↑ Low-frequency ipsilateral TENS↔	–	–	Baseline and 12 days after modeling	–
	Somers and Clemente, 2009	Calibrate Semmes-Weinstein monofilaments	High-frequency contralateral TENS↑ Low-frequency contralateral TENS↔	Radiant heat	High-frequency contralateral TENS↔ Low-frequency contralateral TENS↔	–	–	Baseline and 12 days after modeling	–
	Su et al., 2018	von Frey Filaments	High-frequency immediately TENS↓ High-frequency 1-week TENS↔ Low-frequency immediately TENS↔ Low-frequency 1-week TENS↔	Hot-plate test	–	–	–	Baseline, 7, 14, 21, and 28 days	–

**Vera-Portocarrero et al. (2013)* provided the data on the efficacy of TENS following a single intervention.*

TENS, transcutaneous electrical nerve stimulation; ↔, no statistically significant improvement; ↑: significantly improvement; ↓: significantly deterioration.

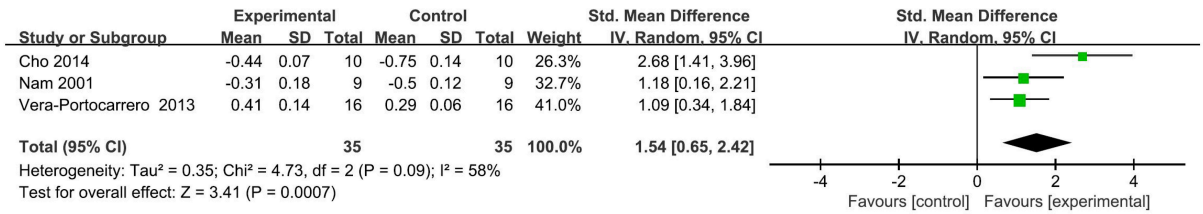

FIGURE 2 | Forest plot of the effect of a single TENS on neuropathic pain versus controls.

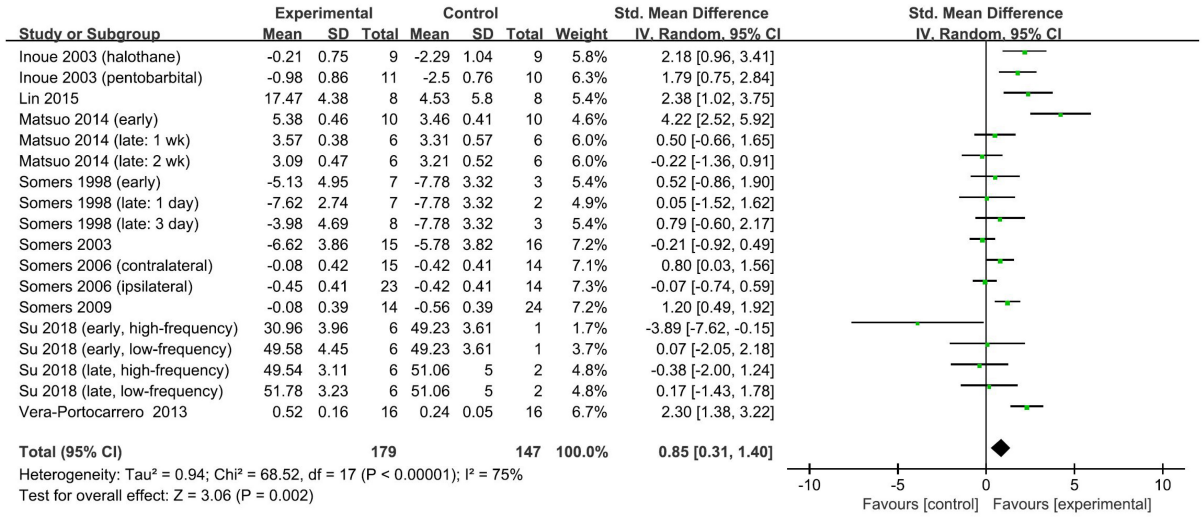

FIGURE 3 | Forest plot of the effect of repetitive TENS on neuropathic pain versus controls.

1.72; $p = 0.004$; $I^2 = 67\%$; **Figure 4**) for studies with the delay of the TENS application. The other predefined stratified analyses were not conducted due to the insufficient number of studies. The overall finding that repetitive TENS significantly ameliorated neuropathic pain persisted in the leave-one-out sensitivity analyses (**Supplementary Material 4B**). Funnel plots were not performed to analyze the publication bias because the number of included studies was small.

DISCUSSION

For the first time, the present meta-analysis included 11 studies that specifically investigated the efficacy of TENS for neuropathic pain in rodent models, and overall, current data suggest that both a single session of TENS and repetitive TENS treatment might temporarily alleviate neuropathic pain in rodent models of neuropathic pain. Of note, the efficacy of repetitive TENS in ameliorating neuropathic pain is not varied by the timing of the application of TENS (no delay or delay). And the results persisted in the leave-one-out sensitivity analyses, which may in part prove the robustness of this meta-analysis. This meta-analysis provides a proof of concept for the application of TENS in pain caused by nerve injury. However, the high risk of bias in the included studies shows that care must be taken when interpreting these findings, and the small number of studies leaves many unsolvable knowledge gaps. Finally, evidence from the review does not support the generalization of the findings of the present meta-analysis to female animals and the long-term nociceptive effect of TENS remains unclear.

The disadvantages of clinical trials are that independent variables and confounders (Volz et al., 2012), including the timing of the application of TENS, cannot be controlled, leading to the insufficient understanding of whether TENS can be utilized in acute or chronic phases of pain caused by nerve injury. To this end, we systematically reviewed animal studies examining the nociceptive effects of TENS and stratified the included studies in terms of the timing of the application of TENS. Data from the present meta-analysis support both the early and delayed application of repetitive TENS, which might provide an appropriate time frame for TENS practice. Specifically, repetitive TENS has the potential to alleviate neuropathic pain in both acute and relatively chronic phases.

The frequency of TENS may be another vital factor that needs to be taken into account, as preclinical studies suggested that high-frequency and low-frequency TENS might exert an analgesic effect through different mechanisms. Studies showed that high-frequency TENS may be mediated *via* δ-opioid receptor class, while low-frequency may work through μ-opioid receptor class and therefore its effects may be limited in people using

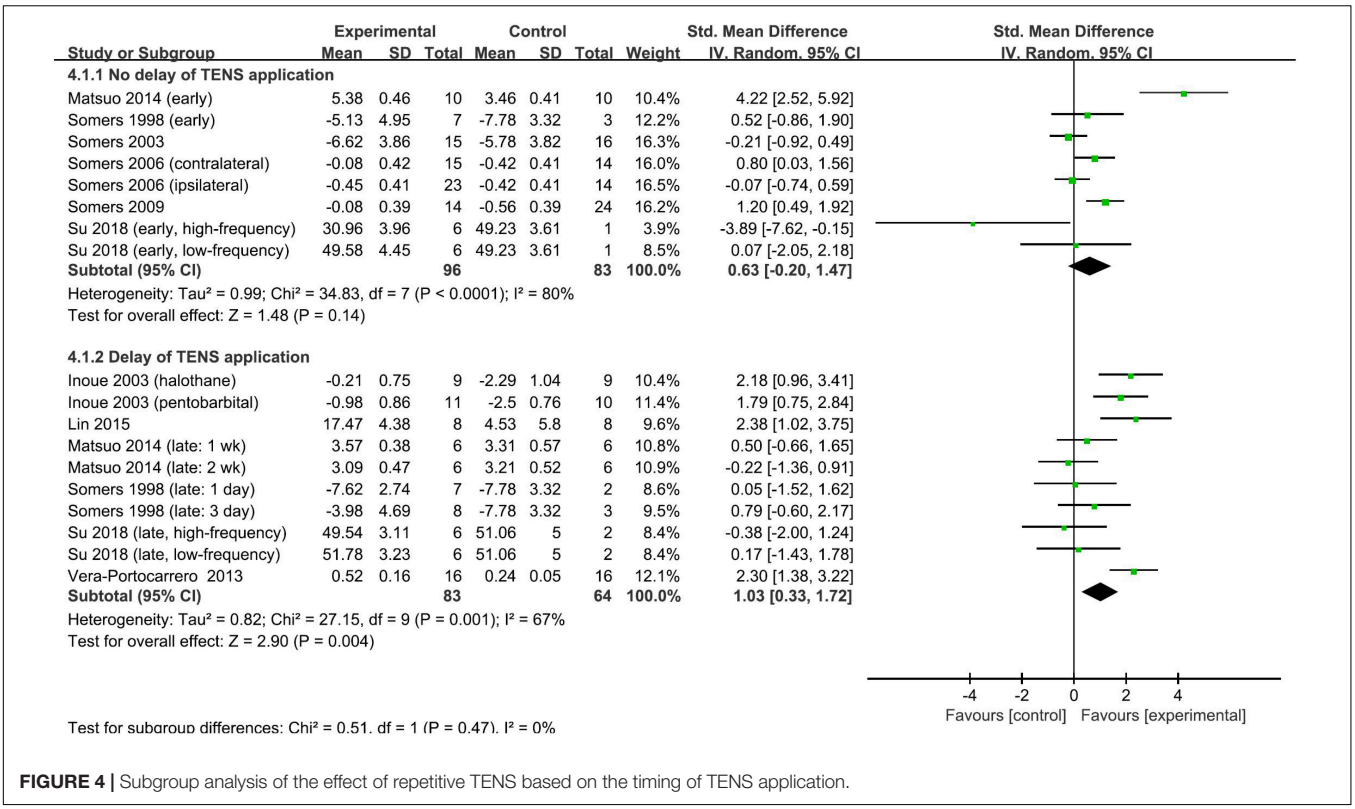

FIGURE 4 | Subgroup analysis of the effect of repetitive TENS based on the timing of TENS application.

opioids, as opioids act through the μ-opioid receptor (Leonard et al., 2010; Sluka et al., 2013; Hendawy and Abuelnaga, 2020). To account for the importance of this, we planned to undertake a subgroup analysis based on frequency. However, studies comparing high-frequency and low-frequency TENS are insufficient to conduct further analysis. Usually, in low-frequency TENS settings, the TENS unit delivers low-frequency stimulus at a high stimulus intensity, which is close to the tolerance limit of the individual (Pal et al., 2020). Therefore, the low-frequency TENS is inevitably uncomfortable and is often considered for those who do not respond to high-frequency TENS (Pal et al., 2020). Taken together, it is not surprising most studies utilized high-frequency as an intervention.

Intensity is another crucial factor in maximizing the TENS effect, and it is suggested to maintain the level of intensity throughout TENS procedures by titrating to produce a strong, non-painful sensation (Bjordal et al., 2003; Moran et al., 2011; Sluka et al., 2013; Gibson et al., 2017). A study has shown that the intensity of TENS should be titrated upward to avoid habituation during TENS treatment (Pantaleão et al., 2011). However, all of the included studies did not report on the adjustment of the intensity of TENS during the experiment. Therefore, further animal studies investigating the efficacy of TENS should consider the adjustment of TENS intensity to optimize the efficacy of TENS.

Electrode placement may affect the effect of TENS in ameliorating neuropathic pain. It is found that high-frequency TENS applied contralaterally to the nerve injury better relieves the pain intensity (Somers and Clemente, 2006, 2009), whereas the lack of studies investigating the effect of contralateral TENS prevented the further analysis. Future studies should further confirm the efficacy of contralateral TENS and investigate whether contralateral TENS is frequency-dependent. Once its efficacy is proven, it will provide a useful reference for clinical use in the future.

Anesthesia administrated during TENS procedures requires consideration when investigating the efficacy of TENS as studies showed that anesthetics have properties of increasing (Antognini and Schwartz, 1993; Kingery et al., 2002) or reducing (Drasner, 2001) pain threshold. Furthermore, anesthetized animals during the intervention procedure cannot well mimic the clinical practice of TENS, since humans are commonly kept awake when receiving TENS therapy. Surface electrodes are commonly used in clinical practice; however, it is hard to maintain the placement of stimulation during experiment procedures using this type of electrodes (Chen et al., 2001). Further studies may consider the utilization of implanted electrodes or the development of alternative approaches that might eliminate these technical limitations.

According to the site of injury or disease, neuropathic pain can be classified as peripheral or central pain. Of the included studies, however, all of them used peripheral nerve injury pain models, including CCI, SNI, SNL, and NCI, resulting in whether the findings of the meta-analysis can be generalized to central neuropathic pain (e.g., central post-stroke pain and SCI-induced neuropathic pain) remaining unknown. There is an urgent need to investigate the effect of TENS on central neuropathic pain in future studies.

Sex is also a key factor for pain as both rodent and human studies reported sex differences in the physiologic and anatomical properties related to pain, including the expression and binding of mu-opioid receptor, morphine metabolism, the activation of the immune system, and the descending antinociceptive circuit (Fullerton et al., 2018). However, all of the included studies solely employed male rodents. Consideration must be given to further studies to explore whether the efficacy of TENS is varied by the sex of participants.

Limitations and Strengths

Some limitations inevitably exist in the present meta-analysis. A study limitation is that there might have been several significant heterogeneities in the included studies, such as frequency, duration, and timing of TENS treatment and pain model. For meta-analytic aims, we had to merge these confounding factors in a meta-analysis and, although stratified the studies in terms of the timing of the application of TENS, other factors were analyzed simultaneously due to the insufficient number of included studies. Therefore, a random-effects model that considers this anticipated heterogeneity was utilized in the present meta-analysis. However, to avoid drawing wrong conclusions and thereby gain the most accurate, reliable, and reasonable findings, stratified analyses exploring the influence of the heterogeneity were conducted only if there were three or more comparable studies in a certain index. Another limitation is that the sample sizes of included studies are relatively low, which might restrict the statistical power (Hooijmans et al., 2014a; Huang et al., 2021; Velzen et al., 2021). However, The Principles of Humane Experimental Technique, also known as the "3 Rs," called for every effort to reduce to a minimum the number of animals used in experiments (Flecknell, 2002), as such we would not overcriticize this issue. And fortunately, in meta-analyses of animal studies, it is suggested to focus on the effects direction rather than effect size itself, mainly due to the inevitable heterogeneity (Hooijmans et al., 2014a; Huang et al., 2021). Besides, some included studies did not mention the baseline data of intervention and control groups, which means we had to assess whether different groups were comparable and how this factor may influence the findings. Lastly, the majority of included studies did not or did not adequately describe the information regarding randomization, concealment, blinding, etc., possibly affecting the reliability of the analysis.

Despite these limitations, the present meta-analysis has some strengths. To obtain the most specific results, we included the largest number and most relevant animal studies published hitherto according to the rigorous criteria for inclusion and exclusion, which may be one of the strengths of the present meta-analysis. In addition, to obtain the most reasonable results and thereby help the future animal research design, we utilized the SYRCLE's risk of bias tool for animal studies to evaluate the quality of current studies. Besides, meta-analyses of animal studies can explore the influence of the heterogeneity (Hooijmans et al., 2014a; Huang et al., 2021; Velzen et al., 2021), an important finding of this meta-analysis is that TENS was not a timing-dependent intervention, which may expand the applicability of TENS to some extent.

Implications for Future Research

Future studies should therefore improve the quality of reporting, such as adequately describing the process of randomization, concealment, and binding and providing the sample size calculation of animals, to increase our confidence in estimating the efficacy of TENS. In addition, there is a need for a high quality of study design in future studies. The intensity of TENS should be titrated during the TENS procedures to produce a prespecified stimulation. In terms of outcome measurement, we would strongly recommend all related outcomes be accurately reported at baseline and all measurement times. This would greatly help the future evaluation of effect, including immediately, short-term, and long-term. Valid evaluations of the function should also be a critical reportable outcome in future studies. Safety data and adverse events should also be routinely monitored and reported as secondary outcomes, to explore how an increased stimulation dose of TENS can be reached. Anesthesia-free TENS is needed to develop to simulate the awake state that is maintained in clinical practice. Besides, female animals and central neuropathic pain models used in TENS studies would significantly help us learn more knowledge about the efficacy of TENS in these domains. Lastly, particular attention to study-level moderators and publication bias may augment the ability of research using animal models of neuropathic pain to optimize the efficacy of TENS for neuropathic pain and to know more about the mechanism underlying TENS treatment.

CONCLUSION

The importance of this meta-analysis lies in the demonstration that, for TENS, both a single session and multiple sessions of applications lead to temporarily ameliorating the pain intensity in animal models of neuropathic pain, of which, repetitive TENS treatments are capable of alleviating neuropathic pain in both acute and relatively chronic phases. However, the direct extrapolation of the animal data to clinical practice is tenuous due to methodological limitations. Particular focus on the quality of TENS study design and reporting may increase the possibility of animal studies to predict the analgesic effect of TENS in humans, thus avoiding more potentially unsuccessful clinical trials, learning more about its therapeutic mechanism, and helping more people with neuropathic pain.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JH and YQ conceived and designed the study. JH, CY, and TW developed the search strategy. JH, ZZ, and YQ screened abstracts

and full text reports. JH, YC, and YQ extracted outcomes. JH and KZ interpretation of the data. JH and CY wrote the manuscript. All authors contributed to the article and approved the submitted version.

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A Bibliometric Analysis of Research on Ketamine From 2001 to 2020

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Background: Ketamine is an intravenous anesthetic with analgesic effects that has a rapid onset and short duration of action. Many studies have been conducted on the use of ketamine; however, the quantity and quality of such studies have not been reported. Therefore, we aimed to conduct a bibliometric analysis of research on ketamine from 2001 to 2020.

Methods: We used the Web of Science database to get publications on ketamine from January 2001 to December 2020. Various bibliographic information was collected, including the number of publications, year of publication, country of origin, journal name, research hotspots, citation count, and author information.

Results: A total of 5,192 articles were included in the analysis. The United States published the highest number of papers on ketamine and the United States participated in publishing the most papers and disclosure funds. The types of articles in clinical trials were cited more frequently. Most articles on ketamine were published in the journal *Anesthesia and Analgesia*. Furthermore, the antidepressant effect of ketamine has been a research hotspot for the last 20 years.

Conclusion: This study provided a comprehensive analysis of research on ketamine and highlighted the growing interest in ketamine and its antidepressant effects.

Keywords: ketamine, bibliometric analysis, clinical anesthesia, analgesic, depression

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INTRODUCTION

Ketamine is a non-competitive ionized N-methyl-D-aspartate (NMDA) receptor antagonist. NMDA receptors are widely present in the central and peripheral nervous systems. Blocking NMDA channels is the main mechanisms of ketamine' pharmacology effect (Johnson et al., 2015). It is generally believed that ketamine selectively blocks cortical communication system and the thalamo-cortical system, a dissociative anesthesia state in which pain sensation disappears and consciousness may partially exist (Schmid et al., 1999). In addition, ketamine could also promote the endogenous opioid peptides release; affect the metabolism of monoamine neurotransmitters; stimulate μ, δ, and κ opioid receptors; and block Na^{+} and Ca^{2+} plasma channels to exert analgesic effects (Hirota and Lambert, 1996). The effects of ketamine are dose-dependent; in adults, the recovery period after the traditional clinical dose of ketamine for anesthesia is sometimes accompanied by a variety of adverse reactions, such as dreams and hallucinations. Nevertheless, it has become one of the most commonly used basic drugs in pediatric clinical anesthesia because of

its convenient route of administration and less respiratory depression; it is often used for pediatric anesthesia and perioperative analgesia. In addition, the intraspinal injection of ketamine as an auxiliary drug has analgesic and preemptive analgesic effects. Besides, the effect and mechanism study of ketamine on antidepressant is increased gradually, therefore, the overview and publication state on ketamine was analyzed in this study.

Bibliometric analyses can evaluate influential papers in a certain field and objectively analyze their study impact. At present, there is no scientific report on the bibliometric analysis of high-quality and highly cited papers on ketamine. The purpose of our research was to investigate the research hotspots and publication trends regarding ketamine, which helps understand its current research status and provides clinicians with accurate medication standards and new ideas for medication. Using bibliometric methods, 5,192 papers on ketamine from 2001 to 2020 were evaluated and their nature, content, and changes over time were analyzed.

MATERIALS AND METHODS

Search Strategy

We used the Web of Science database to investigate publications on ketamine between 2001 and 2020. We used "ketamine" as the search title, limited the article type to "article or review," and only searched for English publications. We collected the following bibliometric information: year of publication, country, journal, number of citations, authors, funding, disciplines, institutions, and topics. We did not use any exclusion criteria.

Statistical Analysis

The CiteSpace software was used for bibliometric analysis. Statistical analysis was performed using the SPSS software (version 21.0; IBM Corp., Armonk, NY, United States). The data were expressed as mean (range) or percentage. Categorical and continuous variables were analyzed using the χ^2-test and independent-sample *t*-test, respectively. Correlation coefficients (r) and *P*-values were calculated using the Spearman's test. Statistical significance was set at $P < 0.05$.

RESULTS

Year and Country of Publication

In the first 7 years (2001–2007), the number of articles published on ketamine was around 120 per year. From 2008 to 2010, there were no major fluctuations in the number of publications per year. Since 2011, the number of papers published on ketamine has shown an increasing trend (**Figure 1A**). The year with the largest number of papers published was 2020 ($n = 515$). American authors published the highest number of articles on ketamine ($n = 1{,}685$), followed by China ($n = 675$) and Germany ($n = 313$). The average number of citations per article published by British authors was 36.55, followed by the United States (34.81) and France (32.54) (**Table 1**). We also analyzed the cooperation between countries for each published article (**Figure 1B**) and found that research cooperation was highest with the United States. In addition, there were more papers co-authored by Chinese authors than those of other nationalities.

Authors and Institutions

We investigated the top 20 corresponding authors and their institutions according to the number of articles published. The corresponding author with the highest number of publications was Hashimoto Kenji at Chiba University with 46 published papers on ketamine, followed by McIntyre Roger S at the University Health Network in Toronto with 28 publications. We used the H-index to assess the number and level of academic output of the researchers. The highest H-index was that of Hashimoto Kenji (H-index = 24), followed by Zarate Carlos A from the National Institute of Mental Health (H-index = 16). More detailed values are presented in **Table 2**. Next, we analyzed the top 20 institutions; the institution with the highest number of publications was University of California System ($n = 135$), followed by the National Institutes of Health ($n = 124$) and Yale University ($n = 121$). We also analyzed the H-index for each institution; the University of London had the highest H-index (H-index = 131), followed by the National Institutes of Health (H-index = 47) and Yale University (H-index = 43). More detailed data are presented in **Table 3** and **Figure 2**.

Subjects and Funds

We analyzed all the journal disciplines that included articles on ketamine in the past 20 years and compiled statistics on these disciplines. We found that most research on ketamine was published in the discipline of neuroscience ($n = 1{,}363$; 18%) followed by pharmacology and pharmacy ($n = 1{,}054$; 14%) and psychology ($n = 1{,}023$; 13%), as shown in **Figure 3**. We also analyzed the funding agencies mentioned in these articles, and the top 10 funding agencies supporting research on ketamine are shown in **Table 4**. They included three American institutions, two European, Brazilian, and Japanese institutions, and one Chinese institution. Among them, the United States Department of Health and Human Services ($n = 637$) and National Institute of Health ($n = 634$) funded the maximum number of studies were from the United States, followed by the National Natural Science Foundation of China ($n = 289$).

Journal Analysis

Next, we investigated the top 20 journals with articles published on ketamine, as shown in **Table 5**. The top 20 journals were established by the number of articles on ketamine they published during this period. Among them, the journal with the highest number of articles was *Anesthesia and Analgesia* ($n = 129$; each article was cited 46.39 times on average), followed by the journals *Veterinary Anesthesia and Analgesia* ($n = 108$; each article was cited 13.94 times on average) and *Psychopharmacology* ($n = 105$; each article was cited 4.93 times on average). We quantified the number of publications in various journals per year and found that the number of publications in the journals *Veterinary Anesthesia and Analgesia* and *Behavioral*

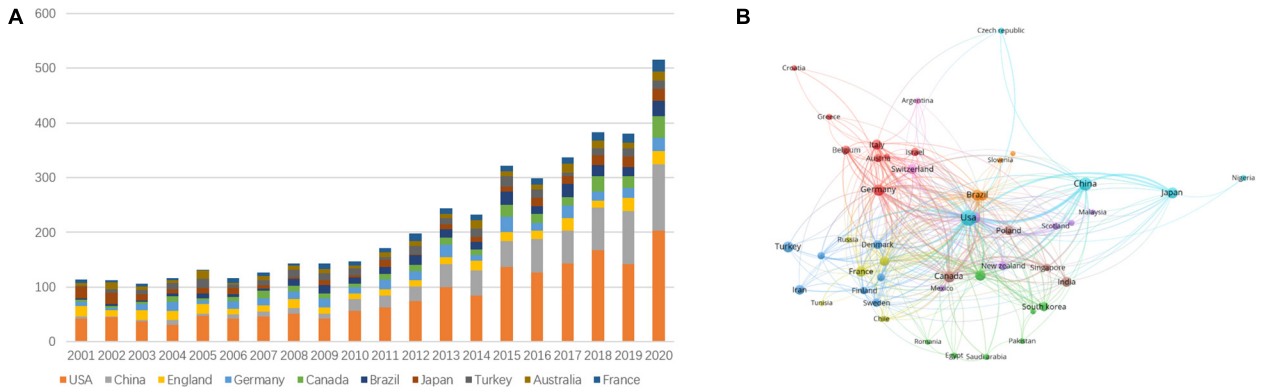

FIGURE 1 | The year and country in which the articles on ketamine were published. **(A)** The number of articles published in different countries each year. **(B)** The country contact map for co-published articles.

Brain Research increased every year in the past 10 years. More detailed data are presented in **Figure 4**.

Citations

In general, the number of citations varied, and we identified the 20 most cited articles. These 20 articles included 9 basic research, 1 review, and 10 clinical research articles. Based on the effects of ketamine, we classified the research content of these articles as follows: 10 articles, antidepressant effects; three articles, antischizophrenic effects; four articles, effects on perioperative pain; and three articles, effects on neurotoxicity (**Figures 5A,B**). The top three cited articles were "Cellular mechanisms underlying the antidepressant effects of ketamine: Role of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors," (766 citations) (Maeng et al., 2008); "NMDAR inhibition-independent antidepressant actions of ketamine metabolites," (724 citations) (Zanos et al., 2016); and "Antidepressant efficacy of ketamine in treatment-resistant major depression: A two-site randomized controlled trial," (621 citations) (Murrough et al., 2013). We found that the top four cited papers were all related to the antidepressant effects of ketamine, and ten out of these twenty articles were on the antidepressant mechanism and clinical applications of ketamine (**Table 6**).

In addition, we analyzed the correlation between the average number of citations, year of publication, and impact factors of the 20 most cited journals; the average number of citations, year of publication ($R = 0.5$, $P < 0.001$, and impact factor ($R = 0.4419$, $P = 0.0014$) were all significantly correlated with one another (**Figures 5C,D**).

TABLE 1 | Number of publications and citations by country.

Countries	Articles	Citations	Average citations per article
United States	1,685	58,655	34.81
China	675	11,101	16.45
Germany	313	8,230	26.29
England	307	11,222	36.55
Canada	262	7,175	27.39
Brazil	256	4,717	18.43
Japan	234	7,013	29.97
Turkey	227	3,413	15.04
France	195	6,345	32.54
Australia	184	4,921	26.74
India	166	2,063	12.43
Italy	144	3,174	22.04
Switzerland	137	3,787	27.64
Iran	117	1,388	11.86
Netherlands	107	4,726	44.17
South Korea	98	1,694	17.29
Poland	85	1,595	18.76
Spain	79	1,253	15.86
Denmark	65	1,774	27.29
New Zealand	64	1,408	22

Research Hotspots and Publication Trends

Research hotspots were identified by the frequency of two keywords that appeared together in the same publication. Additionally, the size of the circles and the thickness of the line represented the frequency of co-occurrence of the keywords. We hypothesized that the hotspots in research on ketamine changed with time; therefore, we classified and summarized all the literature research hotspots every 10 years. From 2001 to 2010, the research interest on ketamine was more about the mechanism of action in neuropathy or brain function, as shown in the red cluster. "Depression" was associated with anesthesia or analgesia, as shown in the green cluster (**Figure 6A**). We found that the research hotspots of articles on ketamine was higher from 2010 to 2020 than in the previous 10 years. "Depression" was the most frequently encountered keyword that appeared with the mechanism cluster (in red), indicating a greater focus on identifying the molecular targets. Our search statistics on the topic of articles in the past 10 years also confirmed that the antidepressant effect of ketamine was the focus of these articles (**Figure 6B**).

TABLE 2 | The 20 authors with the highest number of publications.

Author name	Institution	Number of articles	H-index
Hashimoto, Kenji	Chiba University	46	24
McIntyre, Roger S	University Health Network Toronto	28	8
Dahan, Albert	Leiden University Medical Center	22	9
Ning, YuPing	Guangzhou Medical University, Guangzhou Huiai Hospital	22	8
Su, TungPing	National Yang Ming Chiao Tung University	18	9
Yang, JianJun	Nanjing University, Jinling Hospital	18	9
Zarate, Carlos A., Jr.	NIH National Institute of Mental Health	18	16
Thormann, Wolfgang	University of Bern	15	8
Wang, Cheng	US Food & Drug Administration	15	12
Gao, Li	Northeast Agricultural University	14	6
Kanungo, Jyotshna	US Food & Drug Administration	14	9
Morgan, Celia J. A	University of London	14	14
Reus, Gislaine Z	Universidade do Extremo Sul Catarinense	14	9
Zugno, Alexandra I	Universidade do Extremo Sul Catarinense	14	10
Abdallah, Chadi G	Yale University	13	9
Kuo, HannChorng	Buddhist Tzu Chi General Hospital	13	7
Murrough, James W	Icahn School of Medicine at Mount Sinai	13	13
Kabbaj, Mohamed	Florida State University	12	14
Rodrigues, Ana Lucia S	Universidade Federal de Santa Catarina	12	6
Wainer, Irving W	Cooper Medical School of Rowan University	12	10

TABLE 3 | Top 20 author institutions in terms of number of articles published.

Institution	Articles	Citations	Average citations per article	H-index	Degree centrality
University of California System	135	3,524	26.1	31	234
National Institutes of Health NIH USA	124	8,350	67.34	47	66
Yale University	121	6,592	54.48	43	346
University of London	109	13,1619	25.35	131	90
Harvard University	99	4,279	43.22	35	97
University of Texas System	89	3,633	40.82	29	213
US Department of Veterans Affairs	84	3,781	45.01	35	28
Institut National de la Sante et de la Recherche Medicale Inserm	80	2,896	36.2	28	35
Baylor College of Medicine	61	3,505	57.46	27	139
Assistance Publique Hopitaux Paris Aphp	55	2,553	46.42	26	6
Columbia University	54	2,194	40.63	26	112
Chiba University	53	2,229	42.06	27	62
University of Toronto	52	1,955	37.6	23	258
University of Bern	51	1,146	22.47	22	25
Chinese University of Hong Kong	50	1,003	20.06	20	70
State University System of Florida	50	1,119	22.38	19	31
Icahn School of Medicine At Mount Sinai	47	5,109	108.7	29	173
Mayo Clinic	46	1,494	32.48	20	49
Universidade de São Paulo	45	685	15.22	13	36
University of Pittsburgh	44	1,731	39.34	23	73

DISCUSSION

In this study, we searched for articles on ketamine published in the Web of Science database from 2001 to 2020 and analyzed their basic information. We also conducted a correlation analysis of the articles' citation frequencies. To avoid the differences caused by the year of publication of the article, we chose the average annual citation frequency as a reference indicator and performed a correlation analysis between the year of publication and the impact factor of the articles. The correlation analysis revealed that the articles published later had a higher citation frequency and impact factor. This indicates that ketamine has received increasing attention in recent years with higher numbers of open-access publications on it. Finally, the hotspot trend analysis indicated that an increasing amount of research on

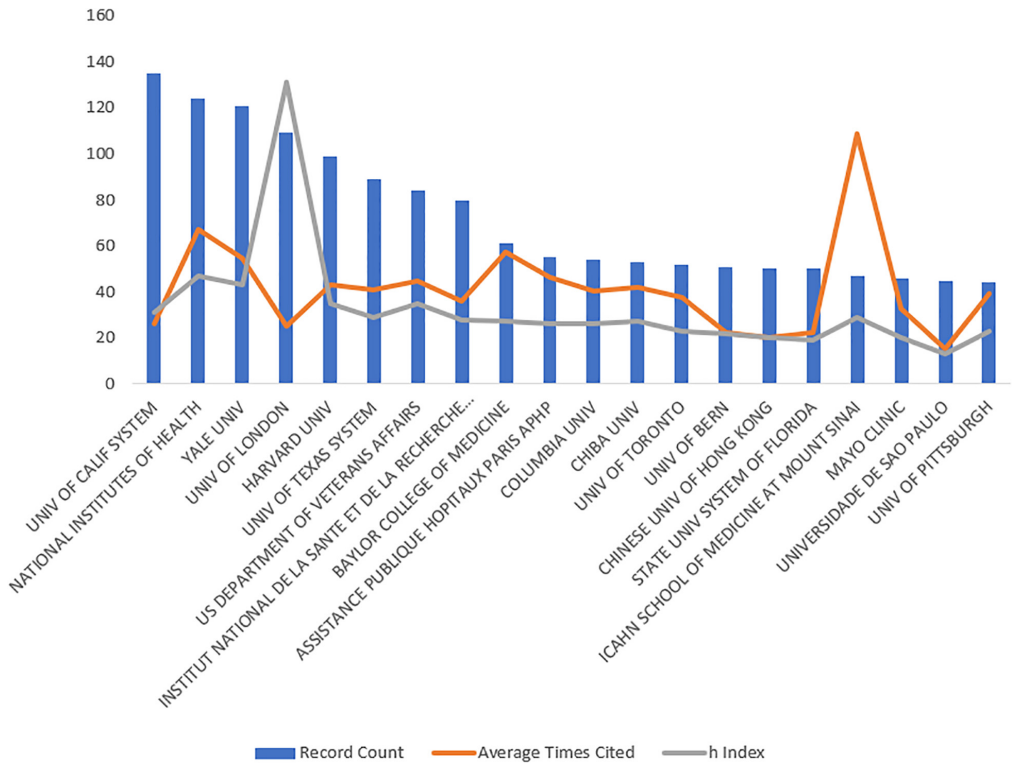

FIGURE 2 | The articles published by different research institutions. The blue bar graph represents the number of articles published by each institution, the red line graph represents the average number of citations per article, and the gray line graph represents the H-index of each institution.

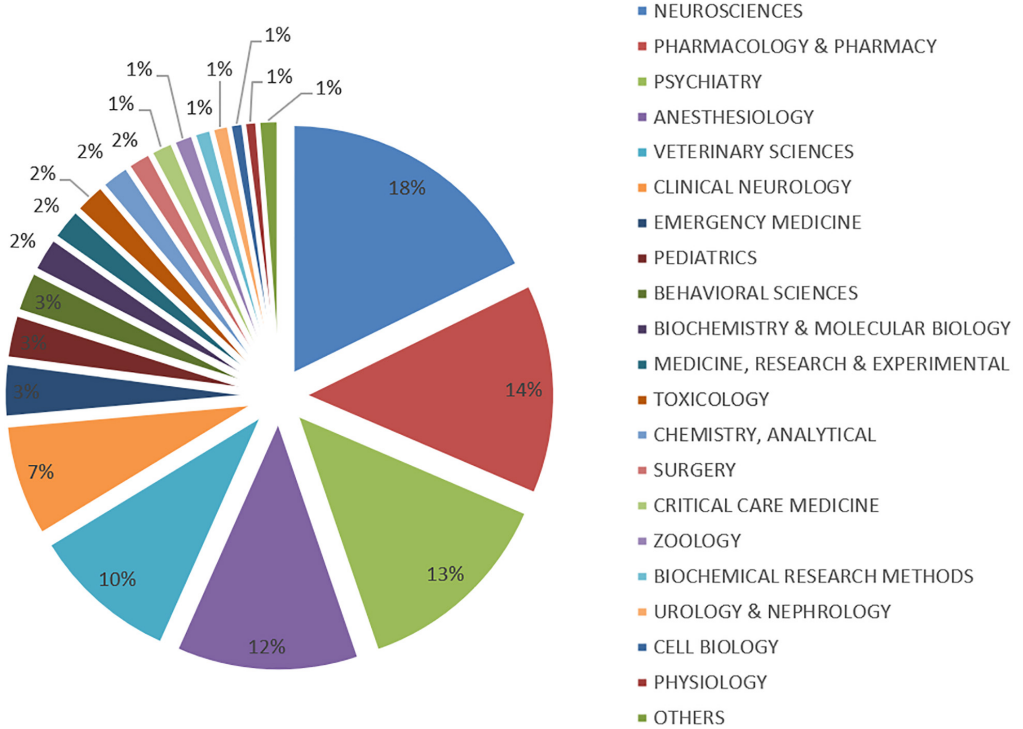

FIGURE 3 | The proportion of articles published in different disciplines.

TABLE 4 | Top 10 funding agencies with publication volume.

Rank number	Funding agency	Number of publications
1	United States Department of Health Human Services	637
2	National Institutes of Health United States	634
3	National Natural Science Foundation of China	289
4	European Commission	253
5	Conselho Nacional de Desenvolvimento Cientifico e Tecnologico CNPq	103
6	NARSAD	86
7	UK Research Innovation	70
8	Coordenacao de Aperfeicoamento de Pessoal de Nivel Superior CAPES	65
9	Ministry of Education Culture Sports Science and Technology Japan	57
10	Japan Society for the Promotion of Science	45

ketamine in the past 10 years has focused on its mechanism as an antidepressant.

The journals wherein articles on ketamine were published gradually changed from *Anesthesia and Analgesia* and *Anesthesiology* to *Behavioral Brain Research* and *Veterinary Anesthesia and Analgesia*. This could be attributed to the fact that ketamine has become a hot topic of research in recent years owing to its antidepressant effects and multiple animal experiments involving it. In terms of co-authorship (**Supplementary Figure 1**), we found that Rosenblat, Joshua D, Nasri, Flora, and Iee, Yena et al. co-authored more articles on ketamine, which may be since they are from the same institution or from the same country. Among the cooperative institutional relationships (**Supplementary Figure 2**), University of California System and university of texas system take great part and co-operative with other institutions frequently. Some other institutions include Guangzhou Medical University, Chiba University, Chinese University of Hong Kong, and Nanjing University were also co-operative closely. The amount of cooperation between these institutions is much higher than that of other institutions, and the number of publications of these institutions is also higher than that of other institutions, which is consistent with our analysis results. By analyzing the correspondence between authors and institutions, we found that Hashimoto, the author with the largest number of papers, and Chiba University, the institution where Kenji works, also published more papers ($n = 53$), ranking 12th, and the author Morgan, who was the 12th author with the same total number of papers. The University of London, where Celia J. A belongs, ranks fourth and Yale University, where the authors of the 15th publishing volume, Abdallah and Chadi G, ranks third in terms of total publication volume. This shows that the institutions of authors with high publication volumes tend to have higher publication volumes, and these authors and institutions are more willing to collaborate with other institutions on co-authoring articles.

In recent years, ketamine has received considerable attention in the treatment of clinical depression, which has aroused a conventional drug in new use; However, this renewed attention might be partly due to an increase in the number of patients with depression in recent years. In addition, more financial disclosures have reflected state and government support for this research. With an aging population, the application and side effects of clinical anesthetics will continue to attract attention. The continuous progress of ketamine research and the strong support of the government has facilitated the developments in the field of anesthesia to a certain extent.

Depression is a common mental illness. Existing antidepressants have a slow onset of action, usually 3–4 weeks, and the failure rate is high (up to 40%) (Thase et al., 2005; Krishnan and Nestler, 2008). Therefore, rapid-acting and effective antidepressants need to be developed; this is a medical problem that needs to be solved urgently. Professor Krystal from the Department of Psychiatry at Yale University School of Medicine and others reported for the first time in 2000 and found that ketamine had a rapid antidepressant effect (Rmbab et al., 2000). A single intravenous infusion of ketamine (0.5 mg/kg) produced an effective antidepressant effect in 4 h and lasted for at least 72 h. Zarate et al. (2006) conducted another randomized double-blind controlled study on patients with refractory depression using the same method of administration and dose, and the results showed that intravenous infusion with a sub-anesthetic dose (0.5 mg/kg) of ketamine improved the symptoms of depression significantly in 110 min after administration. About 71% of the patients showed a significant improvement in their depression symptoms, and 29% of the patients felt relieved 1 day after the administration (Zarate et al., 2006). Price et al. (2009) once again confirmed the rapid and effective antidepressant effect of ketamine and found that it can effectively alleviate or eliminate suicidal ideation in depression patients within 24 h after administration. From 2010 to 2015, Professor Zarate's research team reported a series of research results on the clinical efficacy of ketamine as an antidepressant. These results showed that ketamine could produce rapid, effective, and long-lasting antidepressant effects (Zarate et al., 2012; Ionescu et al., 2015; Moaddel et al., 2015); ketamine not only quickly alleviated the patients' depression symptoms but also attenuated their suicidal tendencies (Ballard et al., 2014). The antidepressant effect of ketamine was first discovered in the clinic, followed by many animal experiments to explore the molecular targets of ketamine and the related mechanism of action. Ketamine has been demonstrated to have significant antidepressant effects in a variety of classic depression models (Maeng et al., 2008; Li et al., 2010; Autry et al., 2011; Beurel et al., 2011; Xu et al., 2013; Zhou et al., 2015).

Several highly cited studies discussed the mechanism and duration of ketamine' antidepressants effect. The study by Maeng et al. (2008) hypothesized that a-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor throughput facilitated ketamine' antidepressant effects. Ketamine was administered at doses of 0.5, 2.5, and 10 mg/kg, and single injections of ketamine can produce rapid antidepressant effects (Maeng et al., 2008). For the persistence of the antidepressant effect of ketamine, Maeng et al. (2008) treated mice with saline, ketamine (2.5 mg/kg) and imipramine (20 mg/kg) and found that only mice in the ketamine group had lower immobility after

TABLE 5 | Ranking of the top 20 journals by citations.

Order	Name	Number of posts	Number of cited	Citations per article	IF	JCR partition
1.	Anesthesia and Analgesia	129	5,984	46.39	5.178	Q1
2.	Veterinary Anesthesia and Analgesia	108	1,506	13.94	1.648	Q2
3.	Psychopharmacology	105	3,673	34.98	4.53	Q2
4.	Anesthesiology	81	4,487	55.4	7.892	Q1
5.	Pediatric anesthesia	75	1,811	24.15	2.556	Q2
6.	Neuropsychopharmacology	68	4,071	59.87	5.251	Q1
7.	British Journal of Anesthesia	58	1,853	31.95	9.166	Q1
8.	Journal of Affective Disorders	58	1,482	25.55	4.839	Q1
9.	Behavioral Brain Research	57	1,279	22.44	3.332	Q2
10.	American Journal of Veterinary Research	56	1,036	18.5	1.156	Q3
11.	Journal of Zoo and Wildlife Medicine	54	425	7.87	0.776	Q3
12.	Neuropharmacology	54	1,664	30.081	5.251	Q1
13.	Pharmacology Biochemistry and Behavior	54	1,197	22.17	3.533	Q2
14.	PLoS ONE	54	1,242	23	3.24	Q2
15.	Biological Psychiatry	49	5,991	122.27	13.382	Q1
16.	Acta Anaesthesiologica Scandinavica	48	1,438	29.96	2.105	Q4
17.	American Journal of Emergency Medicine	47	999	21.26	2.469	Q2
18.	Journal of Psychopharmacology	47	1,502	31.96	4.153	Q2
19.	Neuroscience Letters	47	692	14.72	3.046	Q3
20.	International Journal of Neuropsychopharmacology	43	1,942	45.16	5.176	Q1

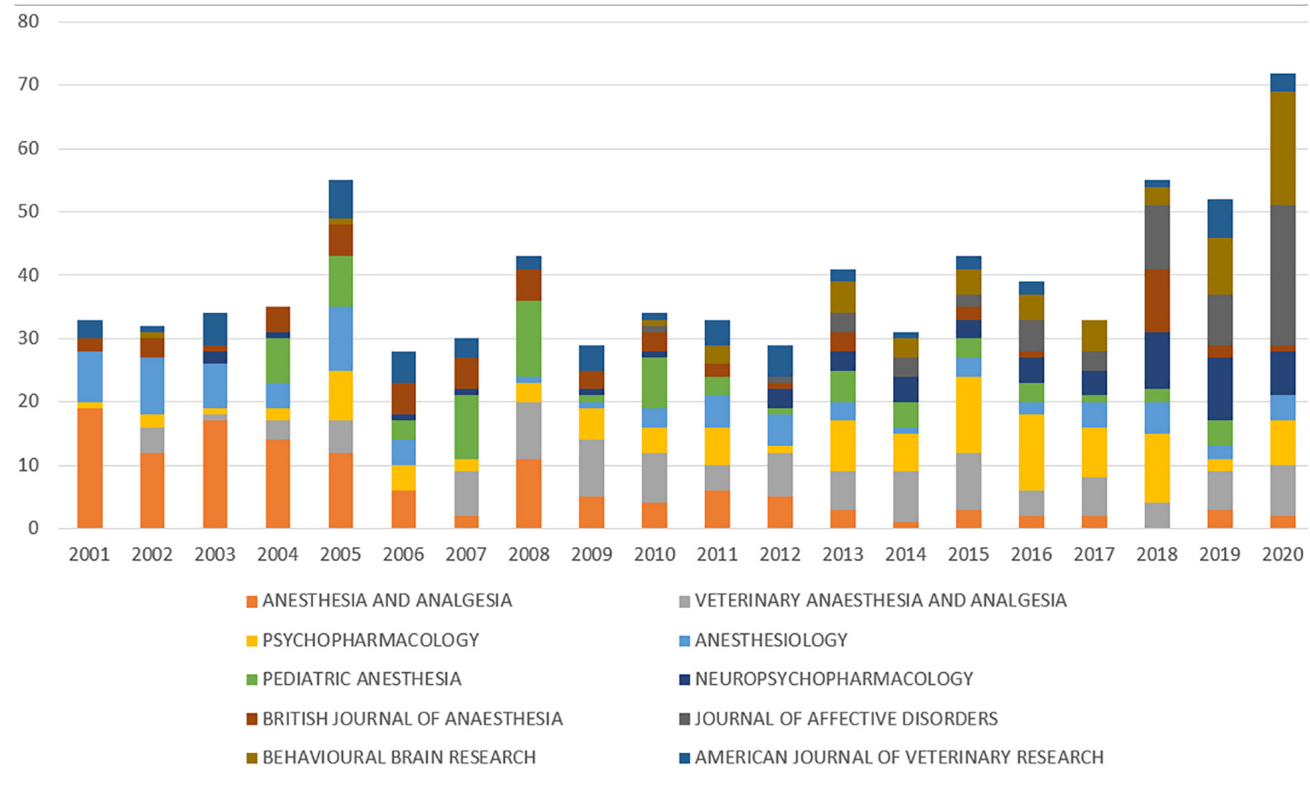

FIGURE 4 | The number of articles published in different journals each year.

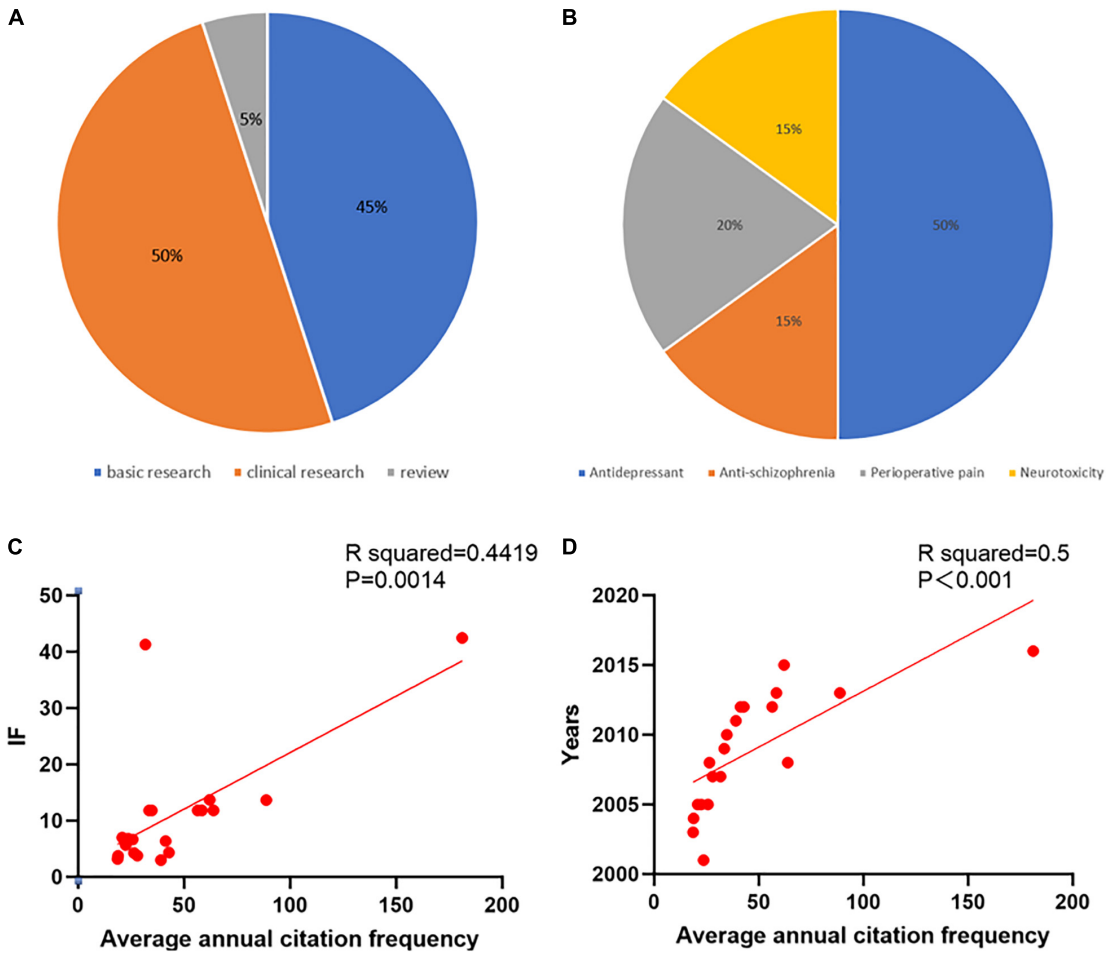

FIGURE 5 | The classification and correlation analysis of the top 20 cited articles on ketamine. **(A)** The proportions of the top 20 ketamine research article classifications. **(B)** The proportions of the research content categories of the top 20 cited articles on ketamine. **(C)** There was a significant correlation between the average number of citations per year and the impact factor based on the correlation analysis (R squared = 0.4419; $P = 0.0014$). **(D)** There was a significant correlation between the average number of citations per year and the year of the analysis (R squared = 0.5; $P < 0.001$).

2 weeks, suggesting that the ketamine' antidepressant effect lasted for a fortnight. The mice were then fear-trained and treated with saline and ketamine and it was found that ketamine did not result in memory impairment. The duration of ketamine induced immobility was shortened after the use of AMPA receptor antagonists. The use of AMPA antagonists significantly blocked the antidepressant effects of MK-801 (a non-selective NMDA antagonist) and Ro25-6981 (a selective NR2B antagonist), and interestingly, neither of them had as long-lasting antidepressant effects as ketamine. Zanos et al. (2016) elaborated that the antidepressant actions of ketamine was NMDA receptors independently, but with AMPA receptor activated. Zanos et al. (2016) also demonstrated the importance of the ketamine metabolite (2S,6S;2R,6R)-hydroxynorketamine (HNK) in the antidepressant effect at the molecular level. Compared with (2S,6S)-HNK from (S)-ketamine, (2R,6R)-HNK derived from (R)-ketamine established more potent antidepressant effect and showed a more pronounced dose dependence than S-ketamine, and that (2R,6R)-HNK showed no significant toxic effects compared to direct ketamine administration, suggesting R-ketamine will be more benefit as a new type of antidepressant drug. In the clinical randomized, double-blind add-on trial, Carlos reported an improvement in depressive symptoms within 3 days after the administration of 0.5 mg/kg ketamine injection for bipolar depression patients, with the most significant side effect being dissociative symptoms, which occurred 40 min after injection (Zarate et al., 2012). In another high-impact clinical research by Murrough et al. (2013) who used 0.5 mg/kg ketamine or 0.045 mg/kg midazolam single infusion in treatment-resistant major depression patients and showed that ketamine had a faster onset of action (dominance ratio 2.18) and higher efficacy (64% for ketamine; 28% for imipramine). Therefore, the difference in study design, dose or disease states did not affect ketamine's antidepressant effect.

Through statistical analysis, we found that more ketamine articles are published in neuroscience. The possible reason is that many basic researches on ketamine have been published in large quantities, to figure out the mechanism of ketamine.

TABLE 6 | Top 20 cited articles.

Rank number	Topic	Corresponding author	Institution	Journal	Year	Cited frequency
1	Cellular mechanisms underlying the antidepressant effects of ketamine: Role of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors	Manji, HK	NIH, Lab Mol Pathophysiol and Expt Therapeut	Biological Psychiatry	2008	766
2	NMDAR inhibition-independent antidepressant actions of ketamine metabolites	Gould, TD	University of Maryland School of Medicine Department of Psychiatry	Nature	2016	724
3	Antidepressant Efficacy of Ketamine in Treatment-Resistant Major Depression: A Two-Site Randomized Controlled Trial	Mathew, SJ	Icahn Sch Med Mt Sinai	American Journal of Psychiatry	2013	621
4	Replication of Ketamine's Antidepressant Efficacy in Bipolar Depression: A Randomized Controlled Add-On Trial	Zarate, CA	NIH, Department of Health and Human Services	Biological Psychiatry	2012	451
5	Effects of ketamine in normal and schizophrenic volunteers	Lahti, AC	University of Maryland School of Medicine Department of Psychiatry Research Center	Neuropsychopharmacology	2001	448
6	Ketamine-induced loss of phenotype of fast-spiking interneurons is mediated by NADPH-oxidase	Behrens, MM	University of California San Diego, Department of Medicine	Science	2007	413
7	Rapid and Longer-Term Antidepressant Effects of Repeated Ketamine Infusions in Treatment-Resistant Major Depression	Murrough, JW	Mount Sinai School of Medicine	Biological Psychiatry	2013	409
8	Remifentanil-induced postoperative hyperalgesia and its prevention with small-dose ketamine	Chauvin, M	Assistance Publique Hôpitaux de Paris	Anesthesiology	2005	386
9	Effects of Intravenous Ketamine on Explicit and Implicit Measures of Suicidality in Treatment-Resistant Depression	Price, RB	Rutgers, The State University	Biological Psychiatry	2009	369
10	Ketamine-induced neuronal cell death in the perinatal rhesus monkey	Slikker, W	U.S. Food and Drug Administration's National Center for Toxicological Research	Toxicological Sciences	2007	363
11	Ketamine anesthesia during the first week of life can cause long-lasting cognitive deficits in rhesus monkeys	Paule, MG	U.S. Food and Drug Administration's National Center for Toxicological Research	Neurotoxicology and Teratology	2011	351
12	Safety and Efficacy of Repeated-Dose Intravenous Ketamine for Treatment-Resistant Depression	aan het Rot, M	University of Groningen	Biological Psychiatry	2010	347
13	Signaling pathways underlying the rapid antidepressant actions of ketamine	Duman, RS	Yale University	Neuropharmacology	2012	343
14	Ketamine and postoperative pain—a quantitative systematic review of randomized trials	Elia, N	University Hospitals Geneva	Pain	2005	337
15	Ketamine use: a review	Curran, HV	UCL, Clinical Psychopharmacology Unit	Addiction	2012	330
16	Acute administration of ketamine induces antidepressant-like effects in the forced swimming test and increases BDNF levels in the rat hippocampus	Quevedo, J	Universidade do Extremo Sul Catarinense	Progress in Neuro-Psychopharmacology and Biological Psychiatry	2008	317
17	Effects of ketamine and N-methyl-D-aspartate on glutamate and dopamine release in the rat prefrontal cortex: Modulation by a group II selective metabotropic glutamate receptor agonist LY379268	Lorrain, DS	Merck Research Laboratories	Neuroscience	2003	317

(Continued)

TABLE 6 | (Continued)

Rank number	Topic	Corresponding author	Institution	Journal	Year	Cited frequency
18	Potential of ketamine and midazolam, individually or in combination, to induce apoptotic neurodegeneration in the infant mouse brain	Olney, JW	Washington University	British Journal of Pharmacology	2005	312
19	Ketamine and Other NMDA Antagonists: Early Clinical Trials and Possible Mechanisms in Depression	Nemeroff, CB	University of Miami	American Journal of Psychiatry	2015	310
20	Ketamine as adjuvant analgesic to opioids: A quantitative and qualitative systematic review	Subramaniam, K	Harvard University	Anesthesia and Analgesia	2004	301

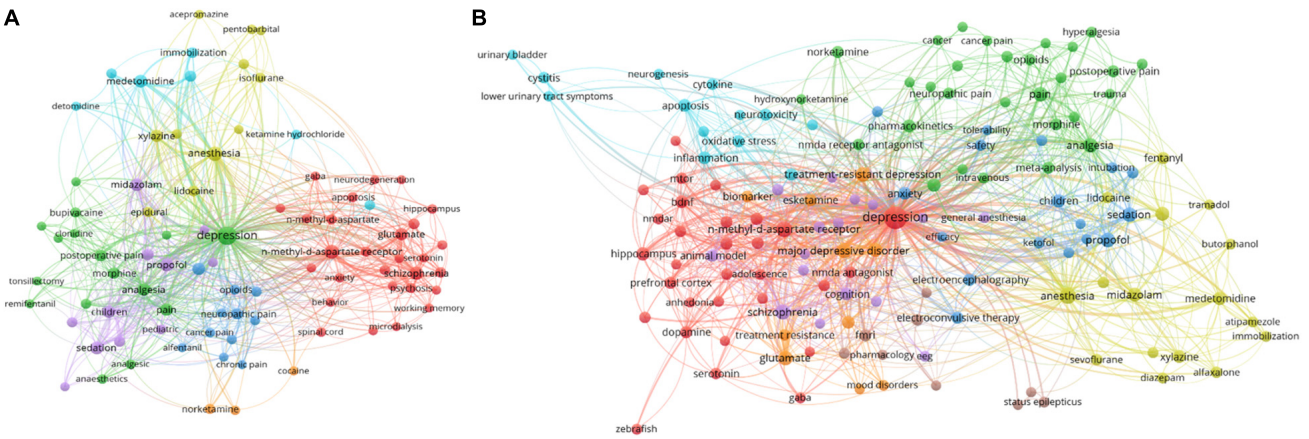

FIGURE 6 | A summary of the hotspot trends of articles on ketamine from 2001 to 2020. The size of the circles and the thickness of the line represent the frequency of co-occurring keywords. **(A)** Hotspots of articles published on ketamine from 2001 to 2010. **(B)** Hotspots of articles published on ketamine from 2011 to 2020.

The following disciplines are pharmacology and psychology list second and psychiatry list third, due to the large number of published studies on the antidepressant effects of ketamine. The focus of different disciplines may also different. For example, the focus of anesthesia may be the application of anesthesia and the use of anesthesia techniques in clinical practice, while the focus of pharmacology maybe the pharmacological effects, toxicological effects, and half-life of drugs themselves. However, some of these disciplines like anesthesiology or neurosciences also intersect with each other, which can participate in both intraoperative anesthesia induction and antidepressant study of ketamine. The differences of topic hotspots or focus keywords among different disciplines would be analyzed in the further study.

Our bibliometric analysis had some inherent limitations. First, some recently published high-impact articles were not included in the "20 most cited articles" because they were not cited sufficiently. However, this does not mean that these articles were not important. Second, we found that articles published in journals with high impact factors tended to receive more attention. In the correlation analysis, we found that the impact factor and the average number of citations of the journal were positively correlated, which showed that high impact factors can inherently lead to bias.

CONCLUSION

We searched and analyzed 5192 English articles published on ketamine from 2001 to 2020. Despite some limitations, our study has found that research interest in ketamine has gradually increased. The research was mostly focused on the clinical application and mechanism of ketamine as an antidepressant, which has also led to more publications on ketamine in mental illness and veterinary journals. Moreover, basic scientific research on the antidepressant mechanism of ketamine has evidently in the past 10 years.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

TZL and HQ conceived, designed the structure of this manuscript, and revised the manuscript. HHM, KY, DYG,

XWL, YC, and XL analyzed and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2022.839198/full#supplementary-material

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Caloric Restriction Alleviates CFA-Induced Inflammatory Pain *via* Elevating β-Hydroxybutyric Acid Expression and Restoring Autophagic Flux in the Spinal Cord

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Inflammatory pain is the most common type of pain encountered in clinical practice; however, the currently available treatments are limited by insufficient efficacy and side effects. Therefore, new methods to relieve inflammatory pain targeting new mechanisms are urgently needed. Preclinical investigations have shown that CR (calorie restriction) exerts analgesic effects in neuropathic and cancer pain; however, the effect of CR on chronic inflammatory pain remains unknown. During calorie restriction, autophagy, a lysosome-dependent degradation process, can be activated to support cell survival. In the present study, we investigated the analgesic effects of CR on complete Freund's adjuvant (CFA)-induced inflammatory pain. The accumulation of LC3-II and p62 showed impaired autophagic flux in the ipsilateral spinal cord of mice with CFA-induced inflammatory pain. CR alleviated mechanical allodynia and thermal hyperalgesia and reduced paw edema and pro-inflammatory factors following CFA administration. CR exerted an analgesic effect by restoring autophagic flux in the spinal cord. Regarding the mechanisms underlying the analgesic effects of CR, β-hydroxybutyric acid (BHB) was studied. CR increased BHB levels in the ipsilateral spinal cord. Furthermore, exogenous BHB administration exerted an analgesic effect by restoring autophagic flux in the spinal cords of CFA-induced inflammatory pain mice. Taken together, these results illustrated that CR relieved inflammatory pain by restoring autophagic flux in the spinal cord, while BHB controlled the benefits of CR, suggesting that CR or BHB might be a promising treatment for inflammatory pain.

Keywords: caloric restriction, CFA, inflammatory pain, β-hydroxybutyric acid, autophagic flux

INTRODUCTION

As a rising health problem, chronic pain is expected to affect up to 30% of adults worldwide (Garland, 2014). Inflammatory pain is the most common type of chronic pain encountered in clinical practice (Barr et al., 2015). Chronic inflammatory pain is characterized by peripheral tissue damage and harmful stimuli that increase the response of the injured site and adjacent tissues,

resulting in symptoms of hyperalgesia and allodynia (Descalzi et al., 2015). Some pro-inflammatory factors, such as interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α), play an important role in the generation and maintenance of inflammatory pain, promoting central sensitization and hyperalgesia (Liu et al., 2021). Inflammatory pain not only seriously affects patients' quality of life, but also creates a huge economic burden (Wyles et al., 2015). Currently available drugs for the treatment of inflammatory pain have various side effects, such as non-steroidal anti-inflammatory drugs (NSAIDs) and opioids. NSAIDs may cause gastrointestinal bleeding, chronic nephritis, and an increased risk of cardiovascular diseases (Lu et al., 2021). The analgesic properties of NSAIDs are insufficient in some patients, and their side effects limit their application in long-term therapy (Grosch et al., 2017). In addition, opioids may cause addiction. Therefore, new therapies to relieve inflammatory pain based on new mechanisms are urgently required.

Autophagy is a vital self-degradative cellular "cleanup" process that facilitates the removal of misfolded or aggregated proteins, as well as recycling of damaged cell components (Bagherniya et al., 2018). Autophagic flux is defined as the progression of autophagy, from the formation of autophagosomes to cargo delivery and lysosomal degradation by proteases. LC3 is cleaved from LC3I into a lower molecular weight LC3II and aggregates on to autophagosome membranes during the autophagy process. LC3II has been shown to be degraded for recycling during the last stages of autophagy, resulting in decreased levels. Sequestosome1 (SQSTM1/p62) is a protein substrate that is selectively incorporated into autophagosomes and degraded by autophagy. Blockade of autophagy flux is associated with increased p62 levels (Yoshii and Mizushima, 2017). Therefore, the adaptor protein p62 and LC3 are used to measure autophagic flux. Recent studies have shown that autophagy plays an important role in the occurrence and development of neuropathic pain (Liu et al., 2019; Hu et al., 2021; Li et al., 2021). Previous reports have demonstrated the dysfunction of autophagic flux in spinal nerve ligation (SNL), chronic constriction injury (CCI) and the spared nerve injury (SNI) models (Berliocchi et al., 2015). Autophagy interacts with inflammation and is believed to play an important role in inflammatory diseases (Matsuzawa-Ishimoto et al., 2018). Proinflammatory cytokines such as TNF-α and IL-1β are the earliest factors that cause inflammatory pain (Hwang et al., 2019). TNF-α and IL-1β interact with autophagy, which in turn regulates the expression of these proinflammatory cytokines, depending on the cellular context (Ge et al., 2018). Therefore, we investigated how autophagy regulated proinflammatory cytokines and pain behavior in chronic inflammatory pain. Our previous results showed that autophagic flux was impaired in the spinal cord of rats with inflammatory pain. Therefore, restoring autophagic flux may be an important strategy to improve chronic inflammatory pain.

The use of autophagy agonists to systemically enhance autophagy can improve some diseases, but can also cause a wide range of side effects (Yang and Zhang, 2020). Therefore, new strategies with higher efficacy and safety are urgently required. Calorie restriction (CR) activates autophagy and has less impact on animal health. Calorie restriction refers to a 10–30% reduction in food intake compared with *ad libitum* intake in the absence of malnutrition (Lee and Min, 2013). Several studies have shown that CR exerts neuroprotective effects. For example, CR has a significant benefit for prevalent neurodegenerative disorders, such as Alzheimer's disease, Huntington's disease, and Parkinson's disease (Martin et al., 2006). In addition, CR has been reported to be effective in moderating the expression of some inflammatory markers that are upregulated during aging (Ugochukwu and Figgers, 2007). IL-1β and TNF-α are pro-inflammatory mediators known to be released by microglia in the CFA model, therefore, as a molecular correlate of CR efficacy, we tested whether CR was able to reduce it. Additionally, many studies have shown that CR is associated with analgesia. In a formalin-induced acute inflammatory pain model, mice with CR showed reduced pain response (Hargraves and Hentall, 2005), and recent studies have shown that CR can improve neuropathic pain (Liu et al., 2018; De Angelis et al., 2020). However, no studies have yet shown whether CR could improve CFA-induced chronic inflammatory pain. Therefore, we investigated the effects of CR on pain perception and autophagy in mice with CFA-induced chronic inflammatory pain. We used CQ (a lysosomal inhibitor) to block autophagic flux to determine whether CR improves chronic inflammatory pain by restoring autophagic flux. Our data indicated that CR improved CFA-induced inflammatory pain and restored autophagic flux in the spinal cord, and that CQ antagonized the analgesic effect of CR.

The ketone body β-hydroxybutyrate (BHB) is synthesized from fatty acids in the liver and serve as alternative energy sources when the supply of glucose is not enough for the body's energetic need (Newman and Verdin, 2017). BHB levels can be markedly elevated under abnormal conditions such as caloric restriction, fasting, or a low-carbohydrate ketogenic diet, etc. (Huang et al., 2018). A recent study revealed that BHB served as a metabolic intermediary of CR and controlled the benefits of CR for improving ischemia and reperfusion triggered liver injury (Miyauchi et al., 2019). Our data indicated that CR elevated BHB levels in the spinal cord, and BHB might control the benefits of CR in CFA-induced inflammatory pain. In brief, we want to illustrate whether CR can alleviates CFA-induced inflammatory pain by regulating autophagy in the spinal cord and the role of BHB in CR therapy. Our results show that calorie restriction or BHB may be an effective and feasible method to improve chronic inflammatory pain.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (6–8 weeks old) were purchased from Shanghai SLAC Laboratory Co., Ltd. All animals were housed under a 12 h light and dark cycle (temperature, 22–24°C), with free access to water and food. Mice were allowed to adapt to the conditions for at least 7 days before all experiments. All experimental procedures and animal welfare experiments were carried out in accordance with the Ethical Regulation on the Care

and Use of Laboratory Animals of Anhui Medical University, and were approved by the school committee for animal experiments.

Dietary Regimen

A standard regimen was used for CR. All mice received the same commercial laboratory pellets (Xietong Feed Co., Jiangsu, China). Mice in the control and CFA groups were provided feed *ad libitum* (AL). We measured the daily food intake of mice (approximately 3.2 g per day) for 1 week before CFA injection, and mice in the CR and CFA + CR groups received 70% of the average food intake following CFA injection until the mice were sacrificed. Food was weighed and provided to the animals in the CR and CFA + CR groups daily, approximately 1 h before the start of the dark cycle, to avoid disrupting the circadian rhythm (Liu et al., 2018). During this period, body weight was regularly measured (**Figure 1**).

Inflammatory Pain Model

To construct the inflammatory pain model, mice received an intraplantar injection of CFA (20 μl) in their left hind paw. Saline was used as the control instead of CFA.

Drug Treatment

Chloroquine (CQ) was obtained from Sigma-Aldrich (St. Louis, MO, United States) and dissolved in saline for intraperitoneal (i.p.) administration. CQ solution was injected intraperitoneally 1 h before CFA injection at a dose of 50 mg/kg/day, and subsequently administered every day at the same time until the mice were sacrificed (Zhang et al., 2017; Weng et al., 2019). Equivalent dose of normal saline was administered to the CFA + CR group.

DL-BHB (Sigma-Aldrich) was dissolved in PBS and adjusted to a pH of 7.5. BHB was injected intraperitoneally (i.p.) 1 h before CFA administration at a dose of 300 mg/kg/d (3% w/v, 10 ml/kg) and then administered every day at the same time until the mice were sacrificed. The doses of BHB were chosen based on previous studies (Yamanashi et al., 2017; Hazem et al., 2018).

Paw Edema

Paw edema induced by the CFA injection was considered as the paw thickness. We used a digital caliper to measure the maximal dorsal-ventral paw thickness (n = 10–12). Paw thickness was recorded immediately before CFA injection (baseline) and then on days 1, 3, 5, and 7 after CFA injection. The caliper was consistently placed in the center of the left hind paw (Justino et al., 2020).

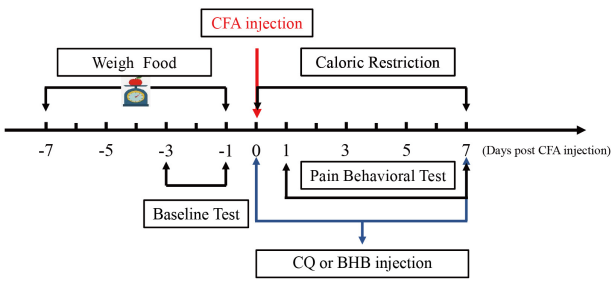

FIGURE 1 | Schematic diagram showing the time points of CR administration and the CQ, BHB injections.

Behavioral Tests

Behavioral tests were performed between 11:00 and 15:00 to minimize any possible influence of the satiety effect of recent feeding and the potential reward of feeding soon after the behavioral test (Liu et al., 2018). Before the behavioral test, the animals were habituated to the testing conditions for 3 days. Mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) tests were performed before CFA injection and on days 1, 3, 5, and 7 following injection (n = 9–12). Before each test, the mice were acclimatized to the surroundings for 30 min. The behavioral investigators were blinded to the drug administration conditions.

In the MWT test, each mouse was placed in a small plexiglass cage (10 × 15 × 15 cm) with a metal mesh floor. Mechanical allodynia was assessed using an electronic von Frey device (2091 series; IITC Life Science Inc., United States). A positive response was defined as flinching or withdrawal of the left hind paw and the force that elicited the withdrawal reflex was recorded. The tests were repeated three times with a 5 min interval between tests, and the mean force was used.

To quantitatively assess TWL, mice were placed on the glass surface of a thermal testing apparatus (Model 336, IITC/Life Science Instruments, Woodland Hills, CA, United States). The movable heat stimulator was moved to focus the heat on the central plantar surface of the left hind paw through the glass plate. Nociceptive endpoints were defined by observation of characteristic lifting or licking of the hind paw, and the time to the endpoint was considered as the paw TWL. Each test session included three thermal stimuli at 5 min intervals, and the mean latency was used. A cutoff time of 20 s was set to avoid tissue damage.

Specimen Preparation

The mice were sacrificed after the behavioral tests. The ipsilateral spinal cord segments (L3–5) were removed and immediately stored at −80°C for subsequent experiments (Xie et al., 2021).

Western Blot

The mice (n = 4) were sacrificed on the seventh day after CFA injection. The ipsilateral spinal cord was mixed with RIPA, and homogenized, then the homogenate was centrifuged at 10,000 rpm at 4°C for 10 min to obtain the supernatant. Proteins extracted from the ipsilateral spinal cord were subjected to 13.5% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. Thereafter, the membrane was incubated in blocking buffer [5% skim milk in Tris-buffered saline with polyoxyethylene sorbitan monolaurate (TBS-T)] for 1 h at room temperature. The membranes were incubated with the following primary antibodies at 4°C overnight: P62 (1:1,000; Abcam), LC3 (1:1,000; Abcam), and Anti- β-actin (1:1,000; Abcam). After washing, membranes were incubated with secondary antibodies (1:10,000;

Bio-Rad) for 1 h at 37°C. The membranes were incubated with ECL reagents and visualized using a chemiluminescence instrument (Amersham Imager 600).

Enzyme-Linked Immunosorbent Assay

Mice (n = 4) were sacrificed on the third day after CFA injection, and the days for enzyme-linked immunosorbent assay (ELISA) were chosen based on previous studies (Zucoloto et al., 2019). The ipsilateral spinal cord was mixed with ice-cold PBS, homogenized, and the homogenate was centrifuged at 10,000 rpm at 4°C for 10 min to obtain the supernatant. The levels of IL-1β and TNF-α were detected using ELISA kits from Cusabio (Wuhan, China), according to the manufacturer's instructions.

Measurement of Spinal β-Hydroxybutyrate Concentration

Mice (n = 4) were sacrificed on the seventh day after CFA administration. Ipsilateral spinal cord samples were rinsed with phosphate-buffered saline (PBS) to remove any red blood cells or clots. Spinal samples were homogenized in beta-hydroxybutyrate assay buffer (Item No. MAK041A; Sigma, United States) and centrifuged at 13,000 × g to obtain the supernatant. The BHB concentration in the supernatant was assayed using a BHB assay kit (Item NoMAK041, Sigma) according to the manufacturer's protocol.

Immunofluorescence Staining

The mice (n = 3) were deeply anesthetized with a lethal dose of sodium pentobarbital (70 mg/kg body weight, i.p.) and perfused with 20 ml PBS, followed by 20 ml of 4% paraformaldehyde on the seventh day after CFA injection. The L3-5 spinal cords segments were removed and post fixed in 4% paraformaldehyde at 4°C overnight. The samples were immersed in 30% phosphate-buffered sucrose for 24 h, and then blocked in Tissue-Tekr OCT compound at −80°C. Immunofluorescence was performed on 8 μm thick L3–5 transverse spinal sections. Sections were collected on microscopic slides, air-dried, and processed for immunofluorescence staining. The spinal cord sections were washed three times for 5 min with PBS, permeabilized in 0.1% Triton X-100 for 10 min, washed three times for 5 min with PBS, and blocked for 1 h with 2% fetal bovine serum (FBS). Subsequently, the spinal cord sections were incubated with primary antibodies at 4°C overnight, LC3 antibody (1:100 Abcam), and then incubated with a AlexaFluor568-conjugated secondary antibodies (1:200, Abcam) for 1 h at 37°C in the dark. Finally, spinal cord sections were observed under a fluorescence microscope (Olympus BX53, Olympus, Japan).

Statistical Analysis

All data are expressed as mean ± *SD*. Statistical analysis was performed using the GraphPad Prism software (version 7.0). Data regarding pain behaviors were analyzed using two-way repeated measures analysis of variance (treatment time), followed by Bonferroni *post-hoc* testing. Data from western blotting, ELISA, and BHB concentration were analyzed using one-way analysis of variance, followed by Tukey's *post-hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered statistically significant.

RESULTS

Calorie Restriction Improves CFA-Induced Inflammatory Pain

MWT and TWL were measured to evaluate the effect of CR on mechanical allodynia and thermal hyperalgesia after CFA injection. As shown in **Figures 2A,B**, CR attenuated CFA-induced mechanical allodynia and thermal hyperalgesia, whereas only CR had no influence on mechanical allodynia and thermal hyperalgesia in control mice. Paw edema was measured to evaluate the anti-inflammatory activity of CR (Almeida et al., 2020). As shown in **Figure 2C**, CFA induced significant left paw edema. Paw edema reached a peak on the first day after CFA administration, and then began to recede gradually in a time-dependent manner. In the CFA + CR group, CR attenuated CFA-induced paw edema. However, CR alone did not change the hind paw thickness in control mice without CFA administration. The levels of proinflammatory cytokines were also measured to study the anti-inflammatory activity of CR. As indicated in **Figures 2E,F**, the concentrations of the pro-inflammatory cytokines IL-1β (**Figure 2E**) and TNF-α (**Figure 2F**) increased in the ipsilateral spinal cord 3 days after CFA injection. In brief, CR administration counteracted the CFA-induced elevation of inflammatory cytokine levels.

Calorie Restriction Ameliorates Autophagic Flux in the Spinal Cord After CFA Administration

To further test the integrity of autophagic flux, sequestosome1 (SQSTM1/p62) and LC3-II were evaluated by western blotting. The results showed that CFA treatment significantly increased p62 and LC3-II levels. Compared with mice in the CFA group, the expression of p62 and LC3-II was decreased in the CFA + CR group (**Figures 3A,B**). In addition, immunofluorescence was used to detect the expression of LC3 in the ipsilateral spinal cord. Compared to the control and CR groups, more LC3 positive cells were observed in the L3–5 spinal cord of mice in the CFA group. However, CR decreased the number of LC3 positive cells after CFA administration (CFA group vs. CFA + CR group) (**Supplementary Figures 2A,B**). These results suggest the blockage of autophagic flux in the ipsilateral spinal cord of mice with CFA-induced inflammatory pain, whereas CR may restore impaired autophagic flux.

Calorie Restriction Improves CFA-Induced Inflammatory Pain by Restoring Autophagic Flux

To determine the exact role of autophagic flux in CFA-induced inflammatory pain, and to determine if CR functions by restoring

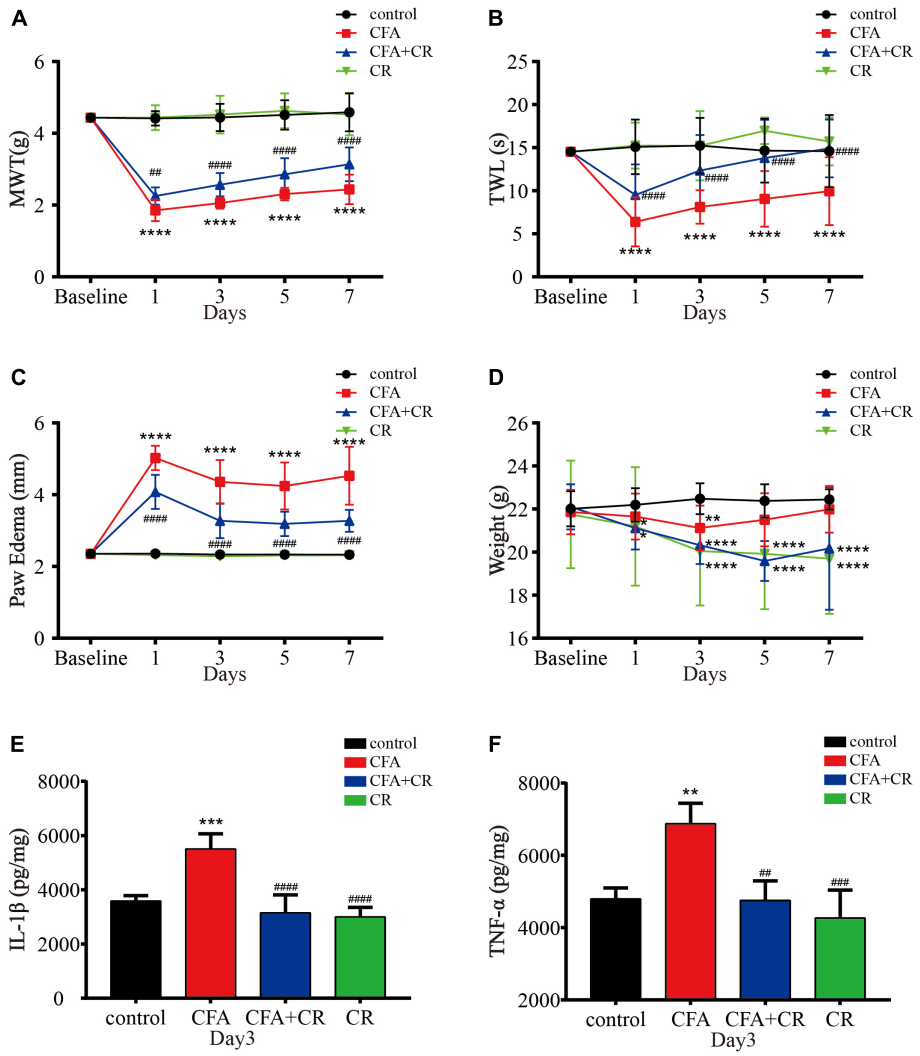

FIGURE 2 | CR attenuates CFA-induced inflammatory pain. **(A)** Results of the mechanical withdrawal threshold (MWT) tests anssd **(B)** thermal withdrawal latency (TWL) tests of mice in the control, CFA, CFA + CR, and CR groups. **(C)** The paw edema and **(D)** the Weigh tests of mice in the four groups. Values are expressed as the mean ± *SD* and were analyzed by two-way repeated measures analysis of variance, followed by Bonferroni *post-hoc* testing, $n = 12$ per group. $^{****}P < 0.0001$, $^{**}P < 0.01$, $^{*}P < 0.05$, compared to control group; $^{\#\#\#\#}P < 0.0001$, $^{\#\#}P < 0.01$, compared to the CFA group. **(E)** The level of IL-1β and **(F)** the level of TNF-α were measured in the four groups. Values are expressed as the mean ± *SD* and were analyzed by one-way analysis of variance, followed by a Turkey's *post-hoc* test, $n = 4$ per group. $^{***}P < 0.001$, $^{**}P < 0.01$, compared to control group; $^{\#\#\#\#}P < 0.0001$, $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$, compared to the CFA group.

autophagic flux, CQ, a lysosomal inhibitor, was used to block autophagic flux. The results showed that CQ co-administration abrogated the decrease in LC3-II and p62 levels induced by CR (**Figures 4A,B**), confirming the blockage of autophagic flux by CQ. Furthermore, by applying the MWT and TWL tests, we found that the MWT and TWL were reduced in the CFA + CR + CQ group compared with those in the CFA + CR group (**Figures 4C,D**), indicating that the analgesic effect of CR therapy on pain perception depended on restoring autophagic flux. Moreover, we measured the levels of pro-inflammatory factors in the spinal cord. The results showed that the levels of IL-1β and TNF-α were significantly higher in the CFA + CR + CQ group than in the CFA + CR group (**Figures 4E,F**). These results demonstrated that CR could improve CFA-induced inflammatory pain by promoting autophagic flux.

β-Hydroxybutyric Acid Controls the Benefits of Calorie Restriction

Next, we explored whether BHB controlled the benefits of CR against inflammatory pain. As shown in **Figure 5A**, BHB levels in the spinal cord were significantly increased in the CFA + CR and CR groups, but CFA administration alone had no impact on the BHB level. These results suggested that CR upregulated BHB levels in the spinal cord. To examine the effect of BHB on inflammatory pain, the mice were injected with BHB. In the CFA + BHB group, BHB levels in the spinal cord

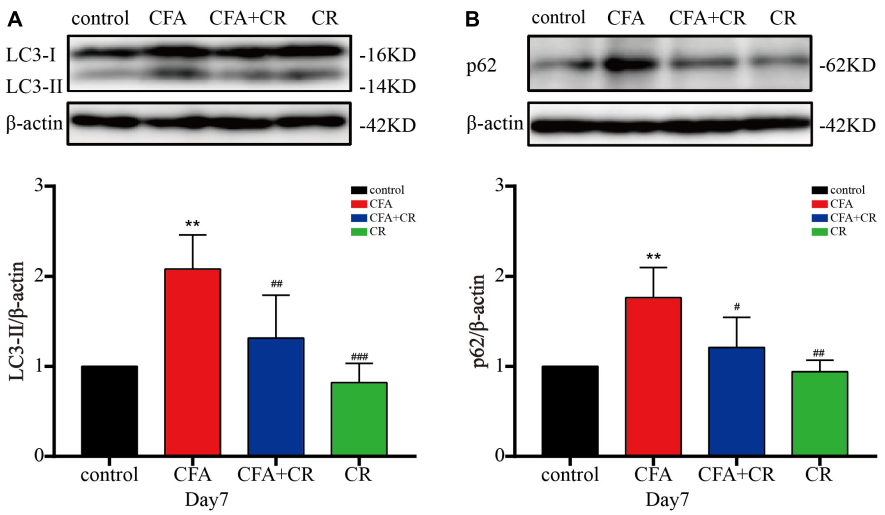

FIGURE 3 | CR ameliorates autophagic flux in the spinal cord after CFA injection. Protein expressions of LC3-II **(A)** and p62 **(B)** of mice in the control, CFA, CFA + CR and CR groups, respectively. Values are expressed as mean ± *SD* and were analyzed by one-way analysis of variance, followed by a Turkey's *post-hoc* test, $n = 4$ per group. $^{**}P < 0.01$, compared to control group; $^{###}P < 0.001$, $^{##}P < 0.01$, $^{#}P < 0.05$, compared to the CFA group.

increased significantly (**Figure 5B**). With regard to autophagic flux, we found that LC3-II and p62 levels were decreased in the CFA + BHB group compared with CFA group, while BHB treatment recovered autophagic flux in the ipsilateral spinal cord after CFA administration (**Figures 5C–E**). As shown above, CR exerted an analgesic effect by restoring autophagic flux in the ipsilateral spinal cord following CFA administration. Therefore, whether BHB also has an analgesic effect on CFA-induced inflammatory pain, its effect on autophagic flux in the spinal cord is similar to that of CR. We found that MWT and TWL were decreased in the CFA + BHB group, and BHB attenuated CFA-induced mechanical and thermal hyperalgesia (**Figures 5F,G**), indicating that BHB improved CFA-induced inflammatory pain. Additionally, we studied the anti-inflammatory activity of BHB against inflammatory pain. In the CFA + BHB group, CFA-induced paw edema was significantly reduced compared to that in the CFA group (**Figure 5H**). The levels of the pro-inflammatory factors IL-1β and TNF-α were significantly decreased in mice administered BHB (**Figures 5I,J**). These results indicate that BHB, as a product of CR, may control the benefits of CR by restoring autophagic flux in the spinal cord.

DISCUSSION

In order to illustrate the role of CR therapy in CFA-induced inflammatory pain, we must illustrate the following questions. First, what is the role of autophagy in inflammatory pain? Second, does CR improve inflammatory pain by regulating autophagy? Third, how does CR regulate autophagy? MWT and TWL were measured to evaluate the mechanical allodynia and thermal hyperalgesia after CFA injection (Zucoloto et al., 2019). Mice treated with calorie restriction were given 70% of their average daily food intake (Zhang et al., 2020). We studied the autophagy by p62 and LC3-II levels to assess autophagic flux (Yoshii and Mizushima, 2017). Our results show that caloric restriction can improve CFA-induced chronic inflammatory pain by restoring autophagic flux in the spinal cord, and BHB might control the benefits of CR. The findings may show promise for treating chronic inflammatory pain.

It has been demonstrated that CR exerts an analgesic effect in neuropathic pain (Coccurello et al., 2018; Liu et al., 2018; De Angelis et al., 2020). Based on this evidence, we designed an experiment to explore whether CR administration has a protective effect against CFA-induced inflammatory pain. Similar to previous studies (Zucoloto et al., 2019), CFA treatment induced significant mechanical allodynia and thermal hyperalgesia. As demonstrated in chronic constriction injury (CCI) model, our results also show that CR exerts an analgesic effect in CFA-induced inflammatory pain. With regard to the anti-inflammatory activity of CR, CR has been reported to be effective in moderating the expression of some inflammatory markers that are upregulated during aging (Ugochukwu and Figgers, 2007). To study the anti-inflammatory ability of CR in chronic inflammatory pain, paw edema and pro-inflammatory cytokine levels were measured (Nguyen et al., 2020; Xie et al., 2021). CR administration significantly reduced CFA-induced paw edema and pro-inflammatory cytokine levels. To gauge the overall health of mice after CR, our results showed that CR decreased body weight (**Figure 2D**), which is similar to the results of previous studies (Liu et al., 2018). These findings indicated that CR improved CFA-induced inflammatory pain and showed obvious anti-inflammatory activity.

Previous reports have demonstrated the dysfunction of autophagic flux in SNL and CCI models (Lipinski et al., 2015; Liu et al., 2017). Therefore, we studied the role of autophagic flux in chronic inflammatory pain. Our study found that LC3-II accumulation was accompanied by a significant elevation

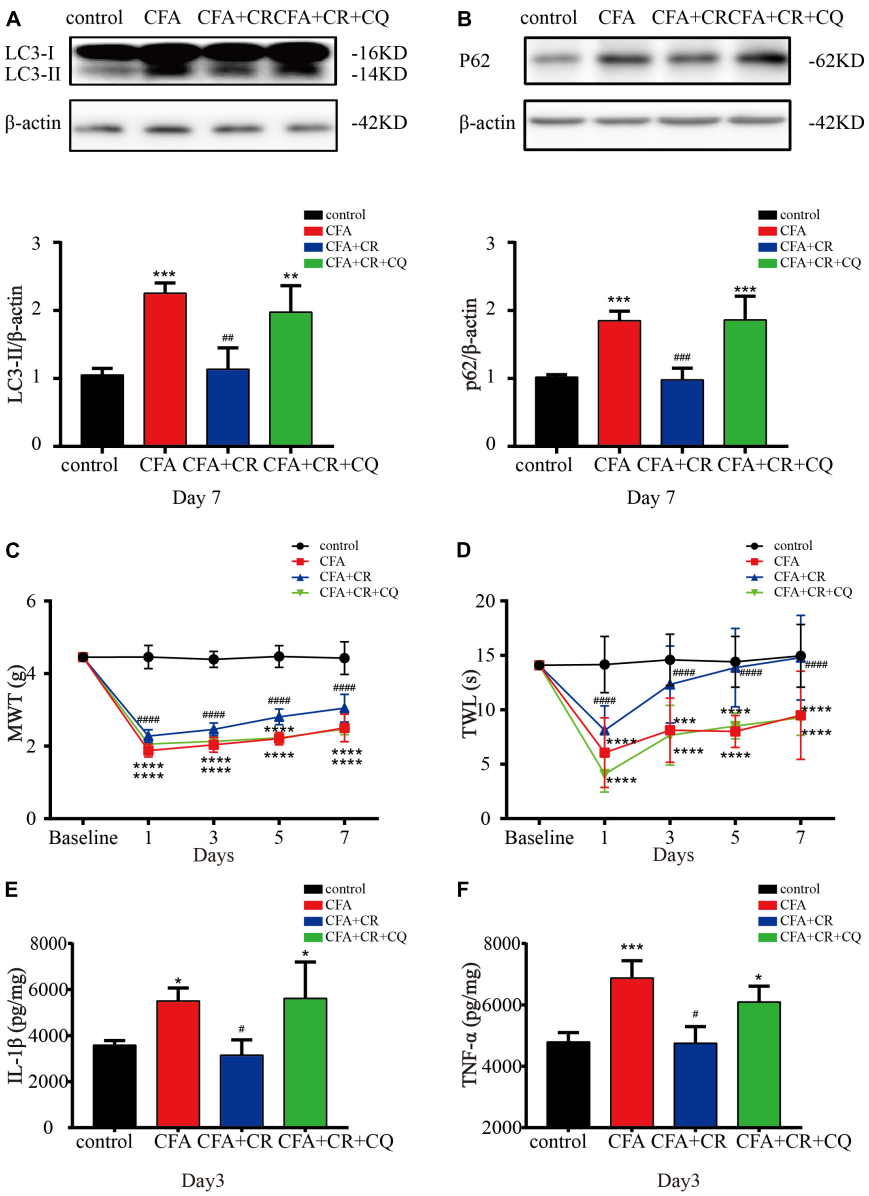

FIGURE 4 | CR improves CFA-induced inflammatory pain by restoring autophagic flux. Protein expressions of LC3-II **(A)** and p62 **(B)** of mice in the control group, CFA group, CFA + CR group and CFA + CR + CQ group. Values are expressed as the mean ± *SD* and were analyzed by one-way analysis of variance, followed by a Turkey's *post-hoc* test, $n = 4$ per group. $^{***}P < 0.001$, $^{**}P < 0.01$, compared to control group;$^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$, compared to the CFA + CR + CQ group. **(C)** The mechanical withdrawal threshold (MWT) tests and **(D)** thermal withdrawal latency (TWL) tests of mice in the four groups. Values are expressed as the mean ± *SD* and were analyzed by two-way repeated measures analysis of variance followed by Bonferroni *post-hoc* testing, $n = 10$ per group. $^{****}P < 0.0001$, $^{***}P < 0.001$, compared to control group; $^{\#\#\#\#}P < 0.0001$, compared to the CFA + CR + CQ group. The level of IL-1β **(E)** and TNF-α **(F)** in the four groups. Values are expressed as the mean ± *SD* and were analyzed by one-way analysis of variance, followed by a Turkey's *post-hoc* test, $n = 4$ per group. $^{***}P < 0.001$, $^{*}P < 0.05$, compared to control group;$^{\#}P < 0.05$, compared to the CFA + CR + CQ group.

of p62 in the CFA group. As simultaneous elevation of the autophagy markers LC3-II and P62 (protein substrates degraded by autophagy) indicated impaired autophagic flux. Moreover, CR has been reported to enhance autophagic flux (Zhang et al., 2020). Consistently, we found that additional CR treatment significantly abrogated the CFA-induced upregulation of LC3-II and p62, demonstrating that CR restored CFA-impaired autophagic flux in the ipsilateral spinal cord. To determine whether CR relieved CFA-induced inflammatory pain by improving autophagic flux, CQ, a classic autophagy-lysosome pathway inhibitor, was used in our study. And the analgesic effect of CR diminished when autophagic flux was inhibited. Our results demonstrated that CR improved inflammatory pain by restoring autophagy in the spinal cord.

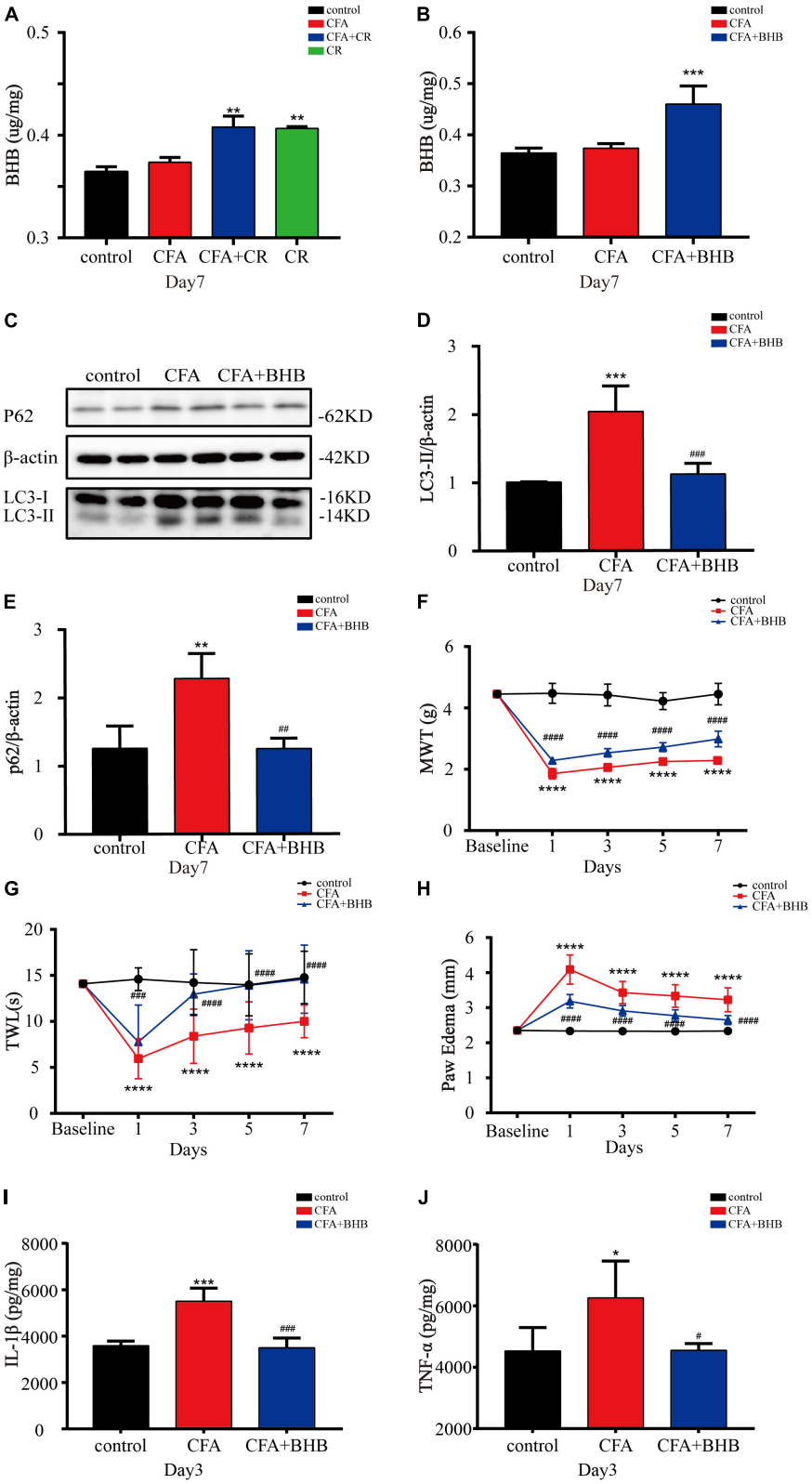

FIGURE 5 | β-Hydroxybutyric acid controls the beneficial effects of CR. **(A)** BHB levels in the spinal cord of mice in the control, CFA, CFA + CR and CR groups. **(B)** BHB levels in the spinal cord of mice in the control, CFA, and CFA + BHB groups after BHB injection. Values are expressed as the mean ± *SD* and were analyzed by

(Continued)

FIGURE 5 | one-way analysis of variance, followed by a Turkey's *post-hoc* test, $n = 4$ per group. $^{***}P < 0.001$, $^{**}P < 0.01$, compared to control group; $^{###}P < 0.001$, $^{##}P < 0.01$, $^{#}P < 0.05$, compared to the CFA group. Protein expressions **(C)** of LC3-II **(D)** and p62 **(E)** of mice in the three groups. Values are expressed as the mean ± *SD*, and were analyzed by one-way analysis of variance, followed by a Turkey's *post-hoc* test, $n = 4$ per group. $^{***}P < 0.001$, $^{**}P < 0.01$, compared to control group; $^{###}P < 0.001$, $^{##}P < 0.01$, compared to the CFA group. **(F)** The mechanical withdrawal threshold (MWT) tests, **(G)** the thermal withdrawal latency (TWL) tests and **(H)** the paw edema tests of mice in the control, CFA, and CFA + BHB group. Values are expressed as mean ± *SD* and were analyzed by two-way repeated measures analysis of variance followed by Bonferroni *post-hoc* testing, $n = 10$ per group. $^{****}P < 0.0001$, compared to control group; $^{####}P < 0.0001$, $^{###}P < 0.001$, compared to the CFA group. **(I)** The level of IL-1β and **(J)** TNF-α were measured in the three groups. Values are expressed as mean ± *SD* and were analyzed by one-way analysis of variance, followed by a Turkey's *post-hoc* test, $n = 4$ per group. $^{***}P < 0.001$, $^{*}P < 0.05$, compared to control group; $^{###}P < 0.001$, $^{#}P < 0.05$, compared to the CFA group.

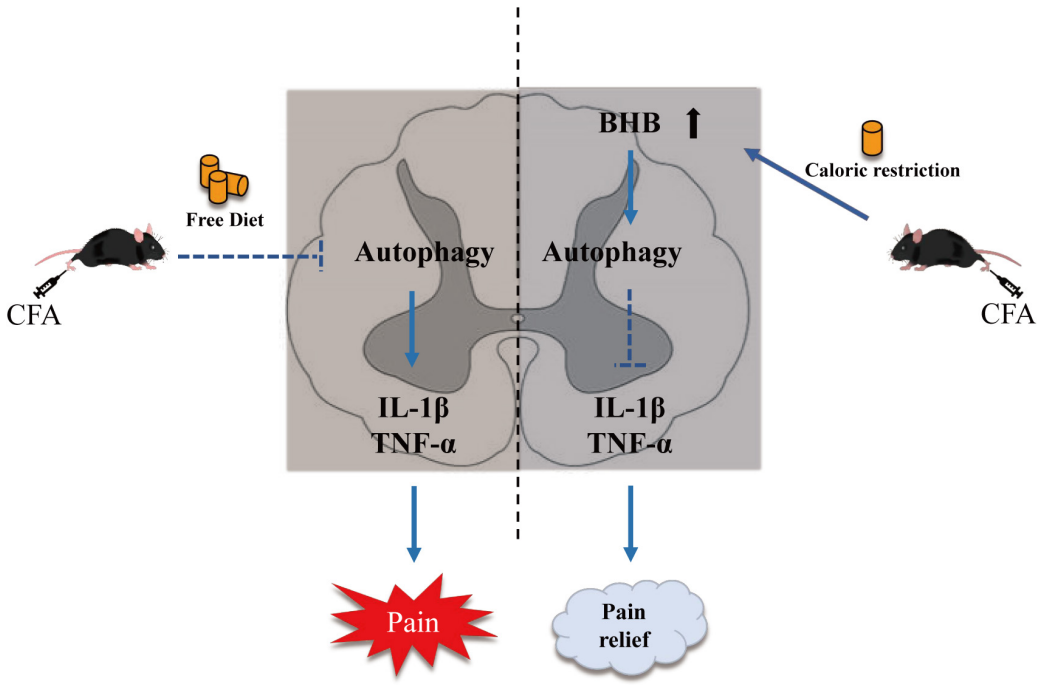

FIGURE 6 | Schematic diagram showing the mechanism by which CR alleviates CFA-induced inflammatory pain. CR elevates β-hydroxybutyric acid expression and restores autophagic flux.

Previous reports have implicated the protective effects of BHB in various neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Tieu et al., 2003; Zhang et al., 2013). BHB has been reported to be effective at improving pain hypersensitivity in SCI models (Qian et al., 2017). BHB also stimulates autophagic degradation during glucose deprivation in cultured neurons (Montiel et al., 2020), and has also been found to stimulate autophagic flux in rats (Habieb et al., 2021). Similar to the results of previous studies (Huang et al., 2018), we observed elevated BHB levels after CR therapy. Therefore, we propose that BHB may control the benefits of CR by restoring autophagic flux in CFA-induced inflammatory pain. To verify this hypothesis, we used the DL-BHB. After intraperitoneal injection of BHB for seven consecutive days, the BHB content in the spinal cord increased. BHB administration improved CFA-induced inflammatory pain, reduced the release of pro-inflammatory factors (IL-1β and TNF-α), and restored autophagic flux in the spinal cords of mice with CFA-induced inflammatory pain.

Many studies have been conducted to elucidate the mechanisms underlying inflammatory pain. The present study showed that autophagic flux might influence inflammatory pain. But little is known about how autophagic flux influences inflammatory pain. Pro-inflammatory factors have a vital role in the induction and maintenance in inflammatory pain (Liu et al., 2021). We found elevated TNF-α and IL-1β levels in CFA-induced inflammatory pain, which we hypothesized to be because of impaired autophagic flux in the spinal cord. Our CQ experiment partially verified this hypothesis. Impairing autophagic flux with CQ ameliorated the analgesic effects of CR. Recent studies have observed crosstalk between autophagy and macrophage polarization. Evidence has shown that autophagic flux is an important mechanism for inducing M2 macrophage polarization, and impaired macrophage autophagy promotes pro-inflammatory macrophage polarization in obese mice (Wang et al., 2018). Microglia are the macrophages of the nervous system. Microglial polarization not only participates in central sensitization, but also affects the secretion of proinflammatory cytokines. Therefore, we will focus our studies on how autophagy regulates proinflammatory cytokines and microglial polarization

to explore the mechanism by which autophagy regulates chronic inflammatory pain in the future.

In conclusion, we observed impaired autophagic flux in the spinal cord of mice with CFA-induced inflammatory pain, and subsequently showed that CR improved inflammatory pain by restoring autophagic flux in the spinal cord. Our results also demonstrated that BHB increased in the spinal cord after CR administration and might control the benefits of CR. CR and BHB may provide potential therapeutic interventions for chronic inflammatory pain (**Figure 6**).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethical Regulation on the Care and Use of Laboratory Animals of Anhui Medical University.

AUTHOR CONTRIBUTIONS

ZY and GX designed the study. CL wrote the manuscript. CL, XZ, LL, YH, QZ, and JZ performed the research and analyzed the data. HW and E-WG revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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ORIGINAL RESEARCH
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Electroencephalogram Mechanism of Dexmedetomidine Deepening Sevoflurane Anesthesia

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Dexmedetomidine, as an α2-adrenoceptor agonist, plays anti-sympathetic, sedative and analgesic roles in perioperative period. Also, dexmedetomidine can reduce the minimal alveolar concentration (MAC) of sevoflurane and the risk of postoperative cognitive dysfunction (POCD) induced by sevoflurane anesthesia. But so far, the electroencephalogram (EEG) mechanism of dexmedetomidine deepening sevoflurane anesthesia is not clear. In this study, by analyzing the changes of the power spectrum and bicoherence spectrum of EEG before and after dexmedetomidine infusion, the EEG mechanism of dexmedetomidine deepening sevoflurane anesthesia was studied. We analyzed dexmedetomidine-induced changes in power spectrum and bicoherence spectrum in 23 patients under sevoflurane anesthesia. After anesthesia induction, the sevoflurane concentration was maintained at 0.8 MAC for 15 min, and then dexmedetomidine was administered at a loading dose of 0.8 μg/kg in 10 min, followed by a maintenance rate of 0.5 $\mu g \cdot kg^{-1} \cdot h^{-1}$. Frontal EEG data from 5 min before and 10 min after dexmedetomidine infusion were compared. After dexmedetomidine infusion, the mean α power peak decreased from 6.09 to 5.43 dB and shifted to a lower frequency, the mean θ bicoherence peak increased from 29.57 to 41.25% and shifted to a lower frequency, and the median α bicoherence peak increased from 41.49 to 46.36% and shifted to a lower frequency. These results demonstrate that dexmedetomidine deepens sevoflurane anesthesia, and enhances α and θ bicoherences while shifting peak values of these bands to lower frequencies through regulating thalamo-cortical reverberation networks probably.

Keywords: dexmedetomidine, cognitive function, sevoflurane, electroencephalogram, anesthesia depth

INTRODUCTION

Dexmedetomidine is an active dextral isomer of medetomidine, which plays anti-sympathetic, sedative, and analgesic roles by activating the α2-adrenoceptor. It is commonly used as a sedative and anesthetic adjunct during perioperative period and can reduce postoperative neurological complications under sevoflurane anesthesia (Gerresheim and Schwemmer, 2013; Keating, 2015). In

practice, the minimal alveolar concentration (MAC) is often used to evaluate the potency of various inhalational anesthetics, and as an indicator of anesthesia depth (Eger et al., 1965). Several studies have shown that dexmedetomidine can reduce the MAC and requirement of sevoflurane (Gozalo-Marcilla et al., 2013; Patel et al., 2013). However, the electroencephalographic (EEG) mechanism of the effect of dexmedetomidine on sevoflurane anesthesia remains unclear.

Spontaneous EEG activity is a commonly used physiological index reflecting the state of consciousness or depth of anesthesia (Freye and Levy, 2005). A growing body of evidence suggests that anesthetics could induce characteristic oscillations by altering or disrupting information processing and communications in the brain (Chauvette et al., 2011; Purdon et al., 2013; Akeju et al., 2014b). These anesthesia-induced oscillations are different as they act on different targets (Musizza and Ribaric, 2010; Purdon et al., 2015b). Dexmedetomidine primarily acts at presynaptic α2 adrenergic receptors to hyperpolarize locus coeruleus neurons by decreasing norepinephrine release (Nacif-Coelho et al., 1994). The reduced release of norepinephrine can result in loss of inhibitory inputs to the preoptic area of the hypothalamus, loss of excitatory inputs to intralaminar nucleus of the thalamus, cortex and decreased thalamo-cortical connectivity (Brown et al., 2011; Akeju et al., 2014a). Sedation under dexmedetomidine is characterized in EEG by spindles and slow-δ oscillations (Huupponen et al., 2008; Xi et al., 2018). Sevoflurane exerts sedative and anesthetic effects mainly through binding at multiple targets in central nervous system including gamma-aminobutyric acid type A receptors ($GABA_AR$), N-methyl-D-aspartic acid receptors (NMDAR), and so on (Nishikawa, 2004). Akeju et al. (2014b) have found that sevoflurane induces coherent frontal α oscillations and slow-wave oscillations to sustain the unconscious state, indicating that sevoflurane may interfere with thalamo-cortical information processing and fragment cortical activity. In view of this, the combination of dexmedetomidine and sevoflurane may produce synergistic or enhancing effects on sedation levels and EEG performance.

Researches continue to validate that α and δ-θ oscillations in EEG induced by anesthetics are associated with neural network regulation and resonance of the thalamo-cortical and cortico-thalamic axons (Schneider and Kochs, 2007; Ching et al., 2010; Akeju et al., 2014b). Bicoherence analysis is a power-independent bispectral analysis that has been developed to detect cross-frequency phase coupling and examine non-linear regulation of brain electrical activities (Hayashi et al., 2008b). In non-linear reverberating system such as seen between the cortex and thalamus, the output signal from the reverberation circuit is reenter into the system as the input signal, leading to self-regulation characteristics and quadratic phase coupling of a variety of different signal wave components (Hayashi et al., 2008b; Araki et al., 2018). Several studies have indicated that bicoherence can reveal the reverberating components and evaluate the electroencephalographic mechanism of combined use of anesthetics (Hagihira et al., 2002; Morimoto et al., 2006; Araki et al., 2018).

In this study, we analyzed changes in EEG bicoherence resulting from dexmedetomidine infusion, and studied the EEG mechanism underlying the effect of dexmedetomidine on sevoflurane anesthesia. We hypothesized that dexmedetomidine deepens sevoflurane anesthesia, and that it changes the power spectra and bicoherence patterns by regulating thalamo-cortical networks.

MATERIALS AND METHODS

Ethical approval for this study (PJ2019-14-17) was provided by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University, Hefei, China (Chairperson Prof. Heng Wang) on November 1, 2019. Written informed consent was obtained from all patients. The trial was registered before patient enrollment at http://www.ChiCTR.org.cn (ChiCTR1900026955).

Study Population

Patients aged 18–65 years with American Society of Anesthesiologists (ASA) physical status 1 and 2 who underwent non-cranial and non-cardiac surgeries were recruited. Patients with dementia, intellectual disability or other neuropsychiatric disorders, severe bradycardia, histories of cerebrovascular disorders, hearing impairment or other factors lead to communication difficulty and those receiving treatment with α2 agonists or antagonists were excluded. Of the 26 enrolled patients, two cases were excluded because EEG data collection was not completed before surgery commenced in accordance with our protocol, one case was excluded due to poor quality EEG. So, twenty-three patients were included in the final analysis. **Figure 1** presents a flow chart of patient selection and exclusion.

Anesthesia Methods

Patients fasted for at least 8 h before surgery and were given no preoperative medication. Standard vital signs (i.e., of non-invasive blood pressure, electrocardiogram, end-tidal carbon dioxide, and pulse oxygen saturation) and EEG monitoring were initiated upon patients' entry into the operating room, and the baseline vital signs were recorded.

Anesthesia was induced with a combination of sevoflurane (6%), sufentanil (0.5 μg/kg), and cisatracurium (0.2 mg/kg). All patients underwent laryngeal mask ventilation, and the sevoflurane concentration was maintained at 0.8 MAC for anesthesia maintenance. After approximately 15 min of stable sevoflurane maintenance (Morimoto et al., 2006), dexmedetomidine (0.8 μg/kg) was administered in a 10 min intravenous infusion. The dose of dexmedetomidine was then changed to 0.5 $\mu g \cdot kg^{-1} \cdot h^{-1}$ for continuous infusion (Harsoor et al., 2014). We maintained intraoperative blood pressure and heart rate fluctuating within 20% of base values. Drugs such as atropine, noradrenaline and ephedrine were used as needed.

Data Collection and Electroencephalogram Preprocessing

Data collection for this study was completed before surgery began. Frontal EEG data were recorded continuously by a SedLine brain function monitor (Masimo Corporation, Irvine, CA, United States) with a sampling rate of 178 Hz and a

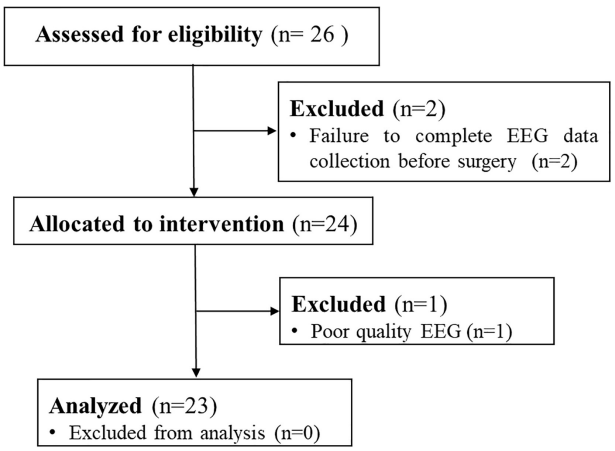

FIGURE 1 | Flow chart of patient selection and exclusion.

TABLE 1 | Characteristics of patients receiving dexmedetomidine infusions under sevoflurane anesthesia.

Characteristic	Mean (SD) or *n* (%)
Age (years)	45.00 (6.68)
Sex (male)	9 (39.13%)
Weight (kg)	58.50 (8.70)
Height (m)	1.62 (0.05)
BMI (kg/m^2)	23.65 (3.07)
ASA physical status	
I	9 (39.13%)
II	14 (60.87%)

BMI, Body Mass Index.

preamplifier bandwidth of 0.5–89 Hz, using the system's standard Sedtrace electrode array [six electrodes at approximately Fp1, Fp2, F7, F8, Fpz (ground), and 1 cm above Fpz (reference)]. Electrode impedance for each channel was $\leq$5 kΩ.

The patient state index (PSI) is a clinically validated measure of monitoring depth of anesthesia and sedation. PSI is a dimensionless number in the range between 100 (fully awake) and 0 (deeply anesthetized) with decreasing values indicating increasing levels of hypnosis (Drover and Ortega, 2006; Buget et al., 2016). The 95% spectral edge frequency (SEF95) is one of the processed EEG measures and the values typically range between 0 and 30 Hz with decreasing values indicating a lower level of responsiveness (Tonner and Bein, 2006). The PSI and SEF95 were recorded every minute.

We applied the linear finite impulse response filter from the EEGLAB toolbox to the raw EEG signals (0.5–50 Hz) (Tort et al., 2008). An experienced investigator visually excluded noise and artifacts. For each subject, we selected two 3-min-long artifact-free EEG segments at 5 min before and 10 min after dexmedetomidine infusion for spectral and bicoherence analysis.

Spectral Analysis

We examined power spectra, defined as quantifications of EEG power at each frequency, and constructed spectrograms, consisting of temporally consecutive power spectra, using the multi-taper method with the Chronux toolbox (Percival and Walden, 1993). The following parameters were used for spectral analysis: window length = 2 s with 0 s overlap, time-bandwidth product = 3, number of tapers = 5 and spectral resolution = 3 Hz. Group-level spectrograms were constructed for the two timepoints by taking the medians across all patients. Group-level spectra and 95% confidence intervals (CIs) were computed by taking median across spectrograms at each timepoint by bootstrap method (Purdon et al., 2015a). Briefly, we resampled spectrogram estimates to obtain replicates and calculated bootstrap group median spectra. We also calculated differences between the group median spectra estimates from the two timepoints for each frequency. The differences were considered significant only when the contiguous frequency bandwidth exceeded the spectral resolution (Akeju et al., 2016). These procedures were repeated 10,000 times, and the percentile method was used to calculate 95% CIs.

Bicoherence Analysis

Similar to the methods used in previous studies (Hayashi et al., 2008a,b, 2010, 2014; Araki et al., 2018), bicoherence at the two timepoints was examined by calculating all pairs of frequencies from 0.5 to 20 Hz at 0.5-Hz intervals, which were represented as two-dimensional moving averages. Nine points of bicoherence were used to calculate diagonal bicoherence (every 0.5 Hz from 1.5 to 20 Hz). The 3-min-long EEG signals were divided into 360 2-s epochs, with 75% overlap, and the Blackman window function was applied. The following equations were used to calculate bicoherence:

Sum of the absolute triple product $[sTP(f_1, f_2)] = \sum_{i=1}^{L} \left|X_i(f_1)X_i(f_2)X_i^*(f_1+f_2)\right|$

Bispectrum $[B(f_1, f_2)] = \left|\sum_{i=1}^{L} X_i(f_1)X_i(f_2)X_i^*(f_1+f_2)\right|$ and

Bicoherence $BIC(f_1, f_2) = 100\frac{B(f_1,f_2)}{sTP(f_1,f_2)}$,

Where j is the epoch number, $X_j(f_1)$ is a complex value calculated by Fourier transformation of the jth epoch and $X_i^*(f_1+f_2)$ is the conjugate of $X_i(f_1+f_2)$. A bicoherent spectrum was represented along the diagonals (the same frequency pairs). The group median diagonal bicoherence (f1 = f2) and 95% CIs were calculated using a bootstrap procedure, with medians of bootstrap samples drawn from the full sample of diagonal bicoherence for each subject at each timepoint. MATLAB was used for all bootstrap calculations (Araki et al., 2018). This procedure was repeated 10,000 times, and the percentile method was used to calculate 95% CIs.

Statistical Analysis

The PSI, SEF95, α power and bicoherence peaks, frequencies at those peaks, and the θ bicoherence peak and their frequencies values from electroencephalogram were compared between 5 min before and 10 min after dexmedetomidine infusion under sevoflurane anesthesia. According to the results of Shapiro–Wilk normality tests of the difference between the two timepoints, the

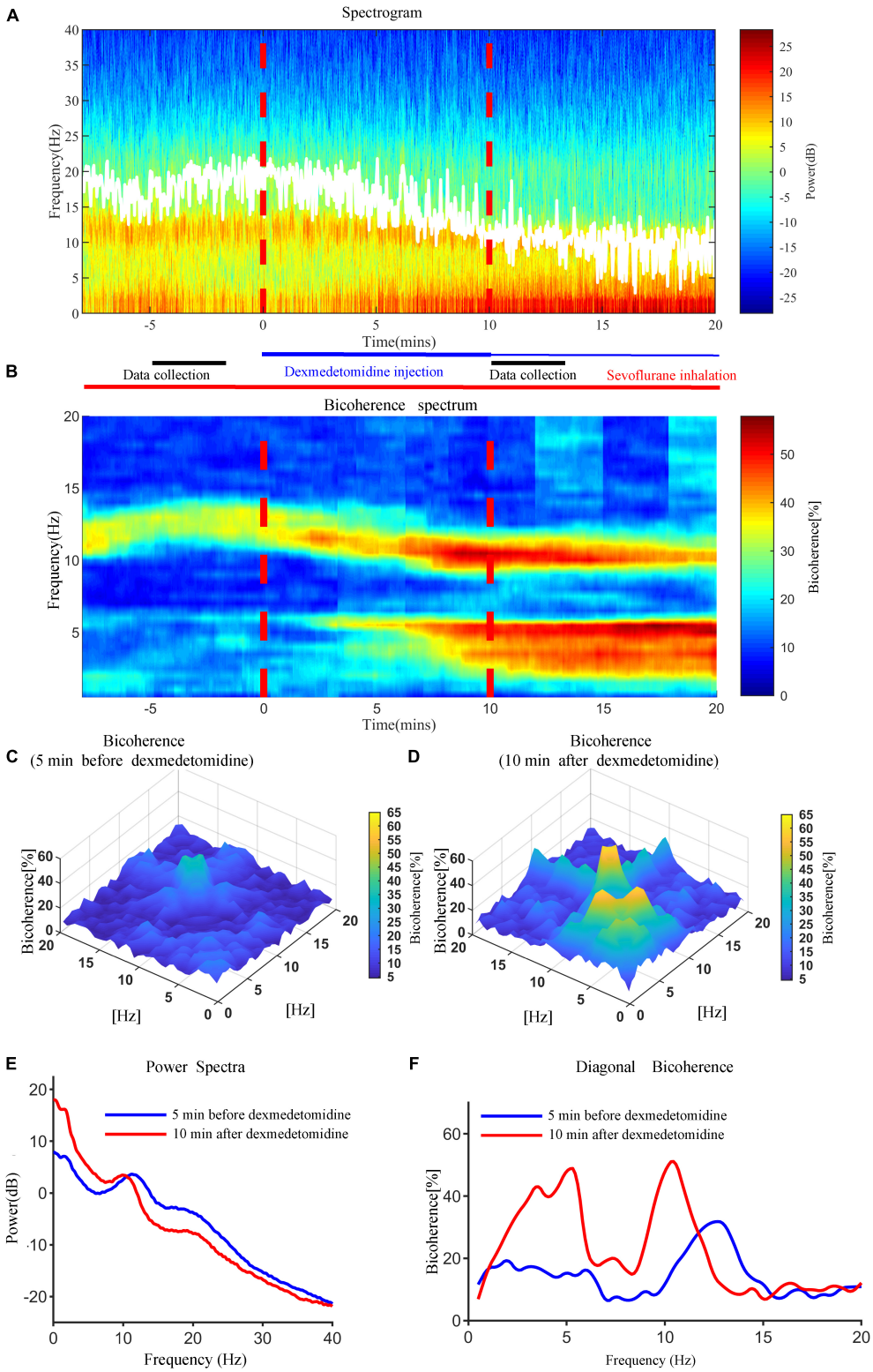

FIGURE 2 | Representative time courses of power and bicoherence spectra 5 min before and 10 min after dexmedetomidine infusion for a 29-year-old man under sevoflurane anesthesia. **(A)** Time-frequency spectrogram of the frontal cortex during anesthesia. **(B)** Bicoherence spectrum of the frontal cortex during anesthesia. White solid lines in **(A)** represent SEF95. **(C)** Bicoherence spectra 5 min before dexmedetomidine infusion for all pairs of frequencies. **(D)** Bicoherence spectra 10 min after dexmedetomidine infusion for all pairs of frequencies. **(E)** Power spectra 5 min before (blue line) and 10 min after (red line) dexmedetomidine infusion. **(F)** Diagonal bicoherence 5 min before (blue line) and 10 min after (red line) dexmedetomidine infusion.

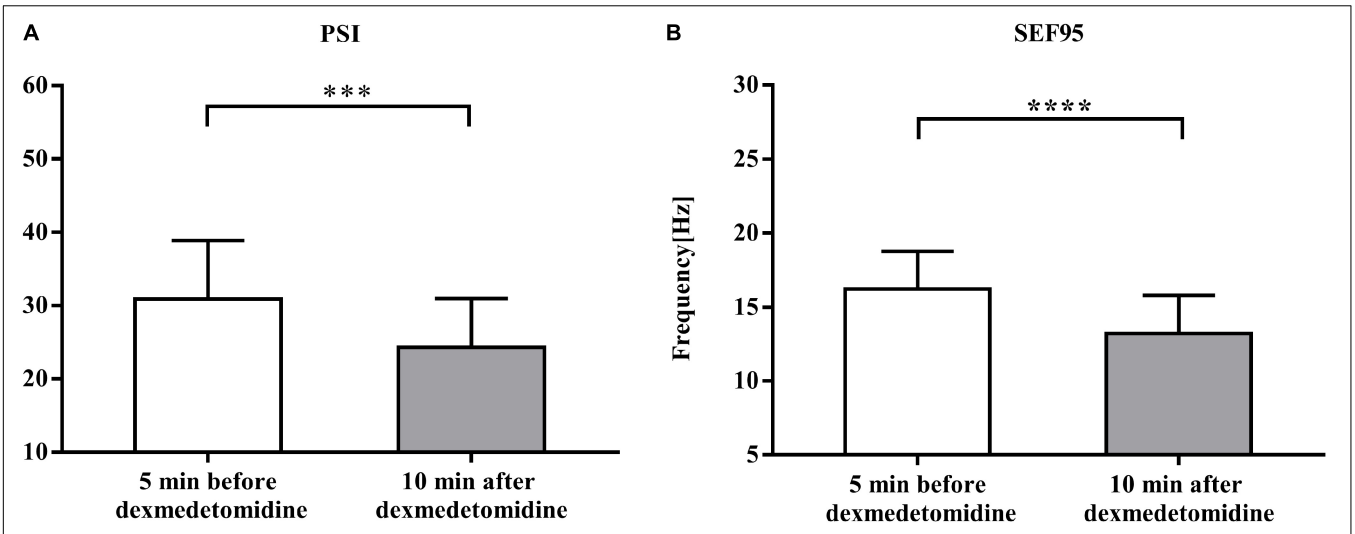

FIGURE 3 | Comparison of EEG monitoring data 5 min before and 10 min after dexmedetomidine infusion. **(A)** Comparison of values of patient status index (PSI) data 5 min before and 10 min after dexmedetomidine infusion under sevoflurane anesthesia. Data are given as mean ± SD (Paired *t*-test probability is indicated as: ***$P < 0.001$; ****$P < 0.0001$, $n = 23$). **(B)** Comparison of values of spectral edge frequency 95 (SEF95) data. Details as in **(A)**.

Wilcoxon signed-rank test (non-normal parameters) or paired *t*-test (normal parameters) were used. The GraphPad Prism software version 5.0 was used for the statistical analysis, and the results were expressed as medians (25th and 75th percentiles) or means ± SD, respectively. Mean/median difference and 95% CIs between groups were calculated by the bootstrap method. $P < 0.05$ was considered to represent statistically significance.

RESULTS

Basic Information

The demographic and clinical characteristics of the 23 patients who participated in this study are presented in **Table 1**.

Representative Time Courses of Power and Bicoherence Spectra

Representative time courses of power and bicoherence spectra before and after dexmedetomidine infusion for a 29-year-old man undergoing meniscoplasty under sevoflurane anesthesia are shown in **Figure 2**. 10 min after dexmedetomidine infusion, EEG activity changed gradually and then achieved the maximum effect; the peak value of θ-band bicoherence increased and moved to a lower frequency, the α-band power peak decreased and moved to a lower frequency, and the bicoherence peak increased and moved to a lower frequency.

The Comparison of Patient State Index and Spectral Edge Frequency 95

Compared with baseline (5 min before dexmedetomidine infusion), the SEF95 and PSI decreased after dexmedetomidine infusion [from 16.24 ± 2.54 Hz to 13.22 ± 2.58 Hz ($P < 0.0001$) and from 30.91 ± 7.98 to 24.30 ± 6.68 ($P < 0.001$), respectively] (**Figure 3**).

Group-Level Spectrograms and Bicoherence Analysis

Group-level spectrograms show that slow-wave power increased after dexmedetomidine infusion (**Figures 4A,B**). The α power peaks decreased [from 6.09 ± 3.45 dB to 5.43 ± 2.90 dB ($P < 0.05$); bootstrap mean difference, −0.66 (−2.46 to 1.16) dB] and moved to lower frequencies [from 10.98 ± 0.78 Hz to 9.92 ± 1.00 Hz ($P < 0.0001$); bootstrap mean difference, −1.06 (−1.56 to −0.56) Hz (**Figure 4**)]. After dexmedetomidine infusion, the θ-band bicoherence peaks increased and moved to lower frequencies [from 29.57 ± 9.14% to 41.25 ± 8.67% ($P < 0.0001$), bootstrap mean difference 11.63% (6.42–16.50%) and from 5.50 (5.50, 6.00) Hz to 5.00 (5.00, 5.50) Hz ($P < 0.0001$), bootstrap median difference –0.5 (–0.5 to 0) Hz (**Figures 5A,D,E**)]. The same pattern was observed for the α-band bicoherence peaks [from 41.49% (30.68%, 49.08%) to 46.36% (39.89%, 54.21%) ($P < 0.001$), bootstrap median difference 4.83% (−2.55 to 14.11%) and from 11.00 (10.50, 11.50) Hz to 10.00 (9.50, 11.00) Hz ($P < 0.0001$), bootstrap median difference –1.00 (–1.50 to 0.00) Hz (**Figures 5B,C**)].

DISCUSSION

The findings of the present study showed that significant decreases in both PSI and SEF95 values after intravenous dexmedetomidine infusion in patients under sevoflurane anesthesia, indicating dexmedetomidine can induce sevoflurane anesthesia from moderate level (PSI value approximately 31) into a deeper level (PSI value approximately 24). After dexmedetomidine infusion, the α power peak decreased and moved to a lower frequency, and the θ and α bicoherence peaks increased and moved to lower frequencies. Previous

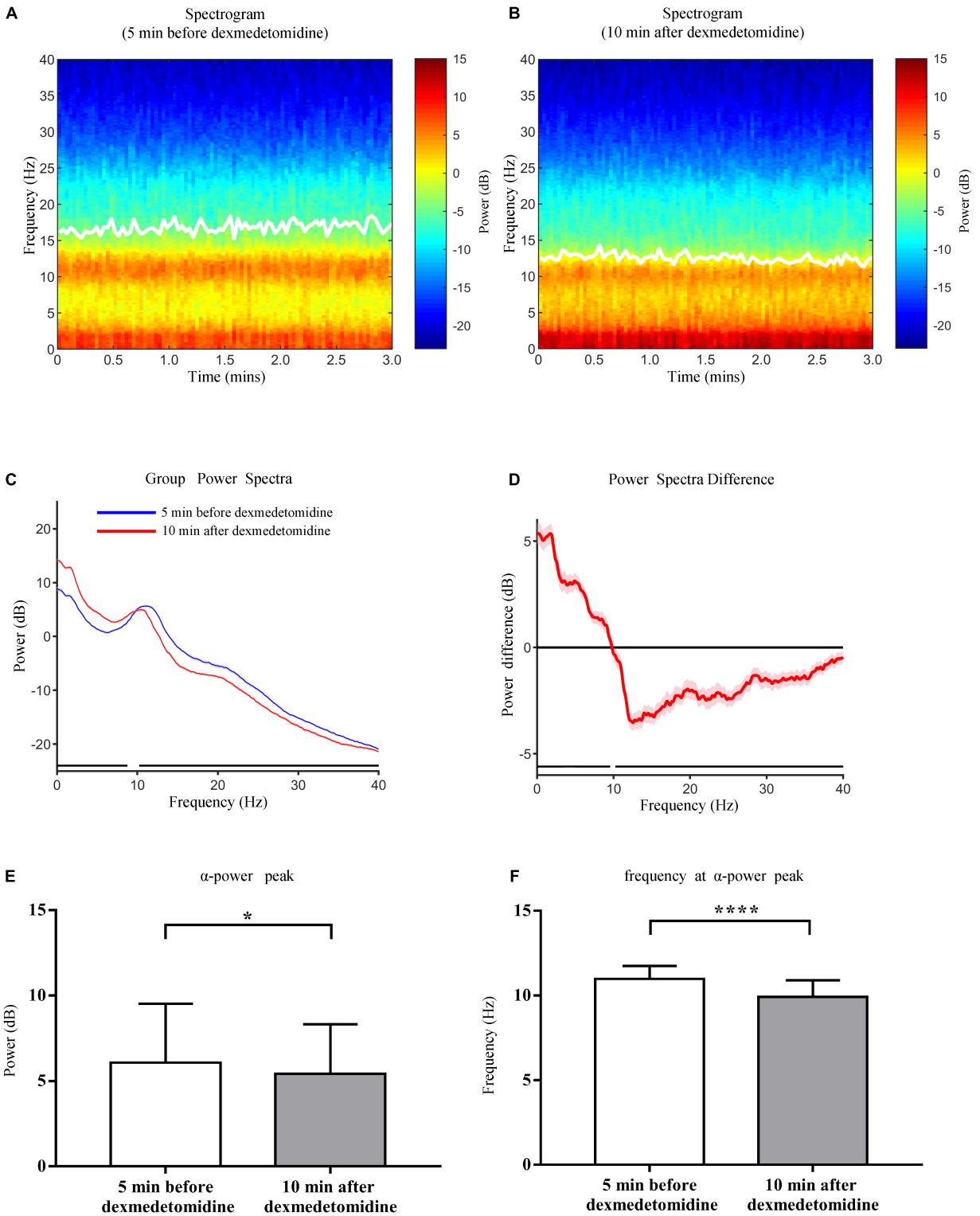

FIGURE 4 | Comparison of group-level power spectral analysis 5 min before and 10 min after dexmedetomidine infusion. **(A)** Frontal spectrogram of 23 cases 5 min before dexmedetomidine infusion. **(B)** Frontal spectrogram 10 min after dexmedetomidine infusion. White solid lines in **(A,B)** represent SEF95. **(C)** Comparison of group-level power spectra 5 min before (blue line) and 10 min after (red line) dexmedetomidine infusion, shading represents 95% CI range. **(D)** Median spectral power difference of two periods at each frequency, shading represents 95% CI range. The horizontal black lines in **(C,D)** represent frequency segments with significant differences across two time periods (0–9.56 Hz and 10.22–40 Hz). **(E)** Comparison of power of α peaks 5 min before and 10 min after dexmedetomidine infusion. Data are given as mean ± SD (Paired *t*-test probability is indicated as: $^{*}P < 0.05$; $^{****}P < 0.0001$, $n = 23$). **(F)** Comparison of frequencies of α peaks. Details as in **(E)**.

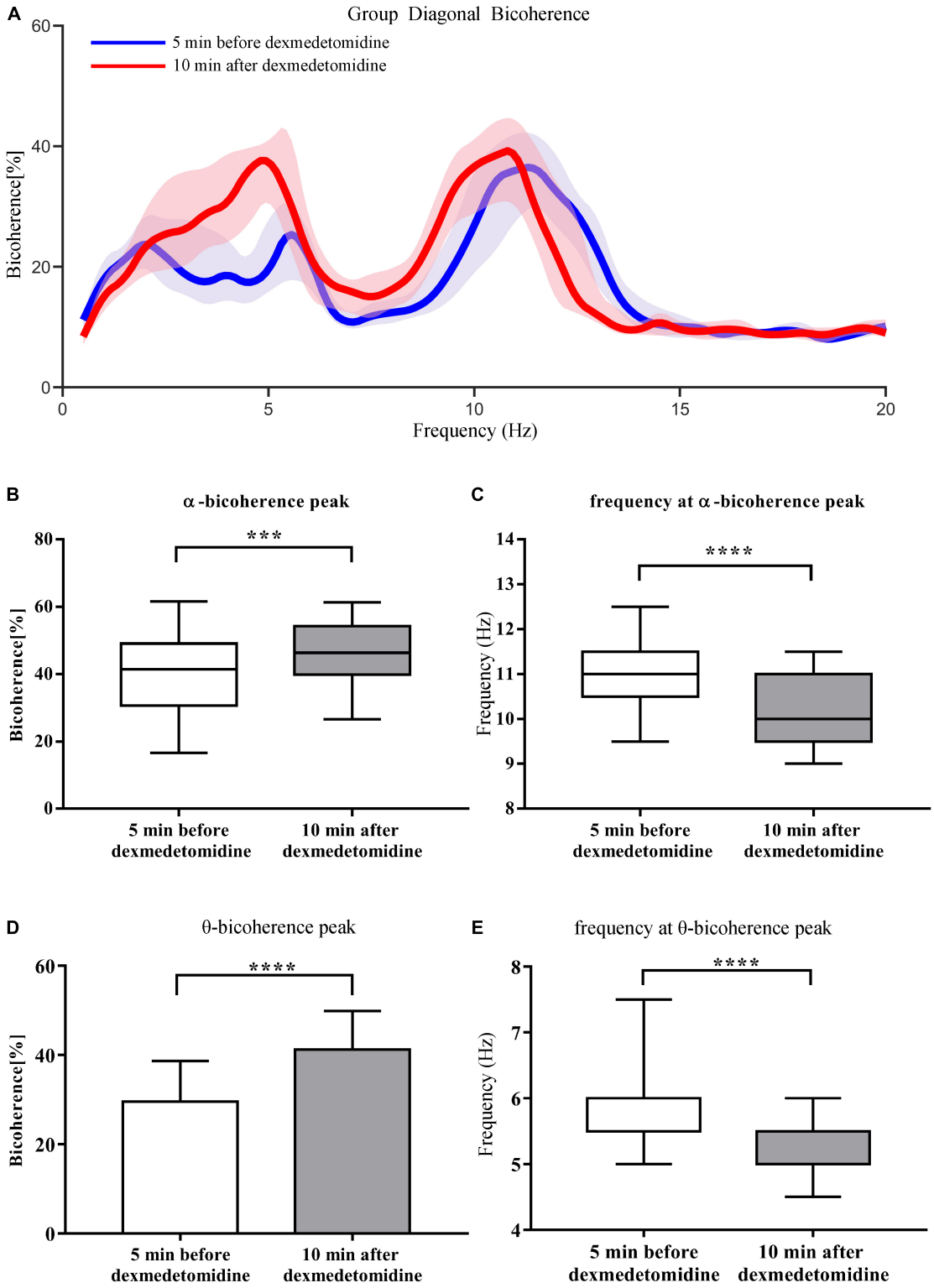

FIGURE 5 | Comparison of group-level diagonal bicoherence analysis 5 min before and 10 min after dexmedetomidine infusion. **(A)** Comparison of diagonal bicoherence spectra from 23 cases 5 min before (blue line) and 10 min after (red line) dexmedetomidine infusion, shading represents 95% CI range. **(B)** Comparison of bicoherence of α peaks 5 min before and 10 min after dexmedetomidine infusion. Data are given as box plots (Wilcoxon signed-rank test probability is indicated as: ***$P < 0.001$; ****$P < 0.0001$, $n = 23$). **(C)** Comparison of frequencies of α bicoherence peaks. Details as in **(B)**. **(D)** Comparison of bicoherence of θ peaks. Data are given as mean ± SD (Paired t-test probability is indicated as: ****$P < 0.0001$, $n = 23$). **(E)** Comparison of frequencies of θ bicoherence peaks. Details as in **(B)**.

studies have found that coherent α and θ oscillations are generally viewed as originating primarily from thalamo-cortical oscillations (Morimoto et al., 2006; Araki et al., 2018). These results indicate that dexmedetomidine augments the effect of sevoflurane anesthesia probably by regulating thalamo-cortical networks.

Neural and Electroencephalogram Mechanisms Behind Spectrograms and Bicoherence Changes

When dexmedetomidine is administered as a low-dose infusion, the EEG shows a combination of slow-δ oscillations with spindles (Huupponen et al., 2008). As the infusion rate of dexmedetomidine increases, the spindles will disappear and the power of slow-δ oscillations will increase. The spindles induced by dexmedetomidine are thought to be generated by thalamo-cortical loop mechanisms (Brown et al., 2011). The slow-wave oscillations induced by dexmedetomidine probably result from decreased excitatory inputs to the cortex and decreased adrenergically mediated excitatory inputs to the basal forebrain, the intralaminar nucleus of the thalamus and cortex (Brown et al., 2011). Similarly, different sevoflurane concentrations can cause different EEG manifestations. When sevoflurane is administered at sub-MAC concentrations, the EEG shows slow-δ oscillations and coherent α oscillations, as the concentration is increased to MAC levels and above, a strong θ oscillation appears, indicates a more profound state of unconsciousness (Akeju et al., 2014b; Purdon et al., 2015b). The EEG changes induced by sevoflurane indicate decreasing frontal and thalamo-cortical connectivity (Ranft et al., 2016). In the current study, we found that after dexmedetomidine infusion under sub-MAC sevoflurane anesthesia, the α power peaks decreased and moved to lower frequencies, and the slow-wave power increased, suggest that dexmedetomidine enhances sevoflurane anesthesia and may be associated with decreased thalamo-cortical connectivity.

The use of bicoherence analysis, as in this study, enables quantification of the degree of phase coupling between signal components and the elucidation of EEG features that cannot be analyzed using simple power spectra (Sigl and Chamoun, 1994; Hayashi et al., 2008b). The signal processing techniques used in previous studies enable the evaluation only of linear processes, thereby ignoring potential non-linear interactions between signal components (Hayashi et al., 2008a). Bicoherence analysis can track changes in non-linear re-input systems, such as that between the cortex and thalamus (Hayashi et al., 2008b). Researchers have reported that when sevoflurane concentration increase from 1% to 3%, α and δ-θ peak frequencies decrease proportionally, and bicoherence in the δ-θ area increases with deepening anesthesia, indicating the obtained features are consistent with characteristics of the thalamo-cortical reverberating networks (Hayashi et al., 2008b). Consistent with their results, we found that α and θ bicoherence increased and their peak frequencies moved to a lower frequency after dexmedetomidine infusion, suggesting dexmedetomidine deepen sevoflurane anesthesia partly by regulating the thalamo-cortical reverberation networks.

Potential Molecular Mechanism of Dexmedetomidine Deepening Sevoflurane Anesthesia

Dexmedetomidine is a highly selective α2-adrenoceptor agonist, which has strong sedative and analgesic effects (Gerresheim and Schwemmer, 2013). α2-adrenoceptors are seven-fold transmembrane receptors belonging to the G-protein–coupled receptor family (Kamibayashi and Maze, 2000). Postsynaptic α2-adrenoceptors exist in many tissues, such as the cerebral cortex and thalamus. Dexmedetomidine activates the G_i protein after interacting with the α2-adrenoceptor (Khan et al., 1999), inhibiting the activity of adenylate cyclase (Afonso and Reis, 2012; Gu et al., 2015; Im et al., 2018). Adenylate cyclase catalyzes the formation of cyclic AMP (cAMP), and cAMP can activate downstream signals as an important second messenger molecule by acting on membrane ion channels. Dexmedetomidine inhibits the voltage-gated sodium channel current by reducing the amount of cAMP, this may be the mechanism by which it deepens sevoflurane anesthesia (Nelson et al., 2001; Gu et al., 2015). The G_i protein also activates potassium ion channels (Khan et al., 1999; Chen et al., 2009), causing cell hyperpolarization, which reduces the activation of excitable cells in the central nervous system, inhibits the discharge of locus neurons (Kamibayashi and Maze, 2000) and inhibits the activity of the norepinephrine pathway. So, dexmedetomidine might regulate the thalamo-cortical reverberation networks through G_i-related mechanisms.

Limitations

There are several limitations in this study. First, the theoretical analysis of the results is based on our extrapolation of the elucidated electrophysiological knowledge, we did not study the brain nuclei associated with the bicoherence and power spectrum changes, or their possible internal linkages or molecular mechanisms. In future, we could use functional magnetic resonance imaging and animal experiments to identify the specific pathways and networks involved in dexmedetomidine-induced deepening of sevoflurane anesthesia. Second, we only assessed frontal EEG, the bicoherence spectrums may differ between different cortical areas (Hayashi et al., 2014), we will use high-density electroencephalogram to explore a more comprehensive mechanism in the future. Third, we did not examine the effect of different doses of dexmedetomidine on sevoflurane anesthesia, which may have yielded different electroencephalogram and molecular mechanisms.

CONCLUSION

After dexmedetomidine infusion during sevoflurane anesthesia, the PSI and SEF95 decreased, the α power peak decreased and moved to a lower frequency, and the θ and α bicoherence peaks increased and moved to lower frequencies. These results revealed

the EEG mechanisms on dexmedetomidine-induced deepening of sevoflurane anesthesia, which might through regulating thalamo-cortical reverberation networks.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LZ conceived the project, supervised the data analysis, and wrote and revised the manuscript. HL collected the data and wrote the manuscript. LD analyzed the data and wrote the manuscript. KF collected and analyzed the data. YC and CH revised the manuscript. EG and JL designed the project, wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Do We Have Measures to Reduce Post-operative Cognitive Dysfunction?

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INTRODUCTION

In the United States alone, over 50 million general anesthetics for surgery are administered every year (Hall et al., 2017). Reduced cognition post-operatively, referred to here as post-operative cognitive dysfunction (POCD), is decreased neurocognitive function after anesthesia and surgery, as compared to preoperative neurocognitive function. It is commonly measured objectively by a battery of neuropsychological tests (Moller et al., 1998). The nomenclature has since evolved under the term of perioperative neurocognitive disorders, which encompass pre-operative impairment, post-operative delirium (POD), as well as cognitive dysfunction within the first 30 days of surgery, known as delayed neurocognitive recovery, and from days 31 to 12 months known as postoperative neurocognitive disorder (Evered et al., 2018; Evered and Goldstein, 2021). While not as emphasized of a risk to patients as the risk of major bleeding, for example, in current practice, the risk of developing POCD is not negligible. For example, in patients undergoing coronary artery bypass surgery on cardiopulmonary bypass, 53% have evidence of POCD at time of discharge, and 24% still have evidence of POCD 6 months later (Newman et al., 2001). When looking at non-cardiac surgery, a remarkable percentage of patients have evidence of POCD at discharge. An early study published in 1999 looked patients over the age of 60 undergoing a variety of orthopedic, urologic, vascular, and abdominal surgeries. Three months after discharge, approximately 10% of patients had evidence of POCD (Moller et al., 1998). A second study confirmed these findings using young, middle, and elderly age groups, with the young and middle-aged groups having a higher number of intra-abdominal and thoracic surgeries, and a lower number of orthopedic surgeries compared to the elderly group (Monk et al., 2008). More importantly, POCD is associated with poor clinical outcomes. There is a correlation between POCD and mortality (Monk et al., 2008), decreased participation in the labor market (Steinmetz et al., 2009), and time to discharge is longer (Silbert et al., 2006). Given these findings, and with an aging population, research into identifying contributing factors to this neurocognitive dysfunction can have huge impacts on our ability to predict its development, and improve how physicians, patients, and families can prepare, prevent, or lessen the risk and severity of its occurrence.

PRE-CLINICAL TO CLINICAL TRIALS

A variety of basic science mechanisms have been proposed for the development of POCD, including a neuro-inflammatory process *via* oxidative stress and microglial activation, mitochondrial dysfunction, synaptic damage, and damage to the blood brain barrier (Cibelli et al., 2010; Cao et al., 2012). The neuro-inflammatory mechanism is proposed to be the major neuropathological process for developing POCD (Zhang et al., 2015; Lin et al., 2020). This has been substantiated with basic science research using aged rats, with histologic immunostaining showing increased inflammation

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in the cortex post-operatively (Zhang et al., 2015). Of course, surgical pain and trauma may play a critical role in the development of inflammatory responses in the peripheral tissues and brain (Lai et al., 2021a).

With the mechanisms behind POCD becoming clearer from basic science research, it is logical to consider interventions for patients. Certain risk factors, such as age, education level, physical state, and preexisting neurovascular diseases, have been associated with an increased risk of POCD, but cannot be practically adjusted (Monk et al., 2008; Lin et al., 2020). More readily adjustable contributing factors have been proposed, including type and depth of anesthetic, ambient noise levels, and the surrounding environment (Zhang et al., 2015; Fan et al., 2016; Berger et al., 2018; Lin et al., 2020). Indeed, the most impactful literature to the practicing anesthesiologist would be the comparison between the two primary types of general anesthetic maintenance methods: total intravenous anesthesia (TIVA) with propofol, and inhaled volatile anesthesia, such as sevoflurane (Zhang et al., 2018; Li et al., 2021). While this avenue of research seems to be the furthest along clinically, the lack of definitive evidence suggests that research into other contributing factors, such as ambient noise levels, and the surrounding environment, needs to continue. Of these, clinical data is relatively lacking, or in its infancy, making these non-pharmacological interventions an intriguing avenue of research.

TYPE AND DEPTH OF MAINTENANCE ANESTHETIC

A study published in 2011 in the *British Journal of Anesthesia* compared the effects in regards to neurocognitive function of a propofol-based TIVA to a sevoflurane anesthetic in patients undergoing on-pump cardiac surgery (Schoen et al., 2011). The basis of the study stemmed from *in vitro* and pre-clinical trials showing that sevoflurane has neuroprotective properties and can lessen ischemia-reperfusion injury in the event of ischemia (Zhang et al., 2015). The study found no differences between the anesthetic types unless the patient had a cerebral blood oxygen saturation level of $< 50\%$. When the levels did decrease to lower than 50%, the patients randomized to the volatile anesthetic group had a lower incidence of cognitive dysfunction (Schoen et al., 2011). While this study only looked at short-term outcomes of 6 days or less, it did provide a link between pre-clinical and clinical trials regarding POCD. To confound the picture, another study of a single center and sub-analysis in 2018 compared the two anesthetic methods in patients aged 65 and older undergoing a variety of cancer surgeries. The study found a higher incidence of POCD in the sevoflurane group compared to propofol-based TIVA one week after surgery (Zhang et al., 2018). Longer-term outcomes, however, were not studied. Most recently, in 2021, a multicenter study was published in *Anesthesiology* in patients 60 years and older undergoing laparoscopic abdominal surgery. Using the definition of POCD put forth by the International Study of Post-operative Cognitive Dysfunction, this study found no differences between the two types of anesthetics 5 to 7 days postoperatively (Li et al., 2021).

Unfortunately, the published studies regarding the differences between the two primary types of anesthesia offer little clarity to this question. While the three studies presented here have mixed conclusions, the differences between the studies, such as patient ages, surgeries, and definition of POCD, make it difficult to compare one study to another. These studies have proposed many more questions than they have answered, laying out a path for future clinical research on POCD, such as using standardized diagnosis criteria for POCD, with a clear stated primary outcome.

Finally, the discussed studies all used processed EEG equipment to monitor the depth of anesthesia with the intention of keeping it similar between groups. Other studies have looked for connections between depth of anesthesia and perioperative neurocognitive disorders, such as POD and POCD. Unfortunately, while processed EEG equipment is certainly much more convenient and simple to use for the practicing anesthesiologist, it is not entirely clear how equivalent it is to interpreting raw EEG wave patterns (Berger et al., 2018). Review of the literature currently does not show clear evidence for routine EEG or processed EEG monitoring during surgery, but clinical trials in this field are expanding and ongoing (Berger et al., 2018; Evered and Goldstein, 2021).

EFFECTS OF NOISE

Noise in the hospital environment consistently exceeds the World Health Organization recommendations (Darbyshire and Young, 2013; Scquizzato et al., 2020). Noise has been proposed to increase the risk of POCD by promoting neuro-inflammation and affecting learning and memory (Lin et al., 2020). Lin et al. showed that mice exposed to a noisy environment showed worsened learning and memory compared to controls 1 day after training sessions. Noise increased inflammatory markers interleukin (IL) 1B and IL-6, as well as microglial marker ionized calcium binding adapter molecule 1 (Iba-1) in the hippocampus, which is important in certain types of learning and memory. Further, the group showed that using minocycline, an antibiotic with anti-inflammatory properties, reduced neuro-inflammation and microglial activation and attenuated the learning impairment (Lin et al., 2020). While this study sheds some light on how noise may play a role in promoting neuro-inflammation, which can impact learning, memory, and increase the risk of POCD, it brings up questions that need to be answered. Nevertheless, caution is needed to extrapolate the findings from this animal study to humans. The mice were subjected to a continuous 75 decibel noise level for 6 h a day, and otherwise housed in a quiet environment, not exactly simulating a hospital environment (Darbyshire and Young, 2013). Interestingly, while the group found a difference in learning and memory for "short-term" memory, defined as 1 day after training sessions, there was no difference in long term memory, defined as 8 days after the training sessions, between the control and study groups (Lin et al., 2020). Human clinical trials on noise are likely not practical, nor ethical. Despite this, it may be possible to assess the incidence and relation between POCD, sleep quality, and noise levels in

hospital ICUs, wards, and short stay units in both short and long term follow up studies.

THE ENRICHED ENVIRONMENT

The perioperative enriched environment is an exciting non-pharmacological research avenue into POCD. The enriched environment (EE), essentially described as immersion into surroundings that lead to social, sensory, and cognitive stimulation, has been shown to increase neurogenesis (Fan et al., 2016). Cognitive enrichment removes certain facilitators of increased physical activity to focus more on the cognitive effects of the environment (Gui et al., 2021). Based on pre-clinical trials, both pre-operative and post-operative environmental enrichment may play a role in the development of POCD.

Fan et al. compared the learning and memory performance of mice immersed in an EE after surgery, to mice in a standard environment both with, and without surgery. The group found that surgery decreased neurogenesis, which was attenuated by exposure to an EE. Learning and memory were also impaired with surgery, as expected, but that the mice exposed to the EE had an attenuated impairment. There was no difference in the learning and memory between mice with surgery exposed to the EE and the control mice that did not undergo surgery. These effects, however, were only seen 1 day after training; whereas there was no difference between the groups in "long-term" memory, defined as 8 days after training (Fan et al., 2016). Kawano et al. in 2015 looked into the effects of pre-operative environmental enrichment in both young and aged rats (Kawano et al., 2015). The group found that 2 weeks of an EE attenuated the negative effects of surgery on cognitive dysfunction and neuroinflammatory markers on aged rats, but not on young rats. Gui et al. in 2021 in part looked at the effect of the EE and the cognitively enriched environment on learning and memory in much older, male mice. Exposing the mice to a cognitively enriched environment post-operatively improved learning and memory for both short- and long-term time periods (Gui et al., 2021), which could be clinically promising in patients unable to perform physical activities. Min et al. in 2021 took the preclinical research a step further and looked for the duration of pre-operative environmental enrichment needed to improve learning and memory of surgery mice. The group could not find a protective effect if exposure to the EE was < 2 weeks, but noted that at 2 weeks of environmental enrichment, both short and long term memory deficits were attenuated (Min et al., 2021). Recently, Lai et al. used adult and old mice to look at the effects of low, medium, and high intensity exercise regimens on neuroinflammation, learning, and memory leading up to surgery. The group found that low intensity exercise attenuated surgery-induced learning and memory deficits. The group suggests that the mechanism for this protection is mediated *via* exercise-induced restoration of healthy gut microbiota and reduction of valeric acid increased and complement 3 signaling activation caused by surgery. These effects ultimately reduce the impairments in learning and memory (Lai et al., 2021b). Not only did this study reinforce the hypothesis of the effects of an EE, but it also shed light onto the mechanisms of how exercise can attenuate surgery-induced cognitive dysfunction.

These studies certainly have potential, as they provide the groundworks for clinical trials. The recently published Neurobics trial (Humeidan et al., 2021) looking at the effect of cognitive prehabilitation on POD serves as a promising link between laboratory and clinical trials. The study found that using cognitive prehabilitation *via* cognitive exercise software was promising in lowering the risk of POD. Thus, similar clinical studies looking at various effects of both pre-, and post-habilitation exercise regimens as well as the effect of cognitive pre-habilitation and rehabilitation on POCD should be pursued. These measures may be applied in susceptible patients to reduce POCD.

In summary, the research into POCD is looking beyond the differences between maintenance anesthetics, which do not appear to make a significant impact on rates of POCD. EEG interpretation intraoperatively, as well as noise level monitoring, may in future be clinically relevant in the hospital environment pending further studies (Darbyshire and Young, 2013; Berger et al., 2018; Lin et al., 2020; Scquizzato et al., 2020; Evered and Goldstein, 2021). Finally, and perhaps most promising, the EE, socially, physically, and cognitively, has yielded exciting early pre-clinical and clinical studies on its effect with delirium and POCD (Kawano et al., 2015; Gui et al., 2021; Humeidan et al., 2021; Lai et al., 2021b; Min et al., 2021). While future studies involving the EE will undoubtedly require rigorous protocols and follow up, the early research has suggested countless of opportunities for clinical trials and perhaps non-pharmacological interventions to reduce the risk and severity of POCD.

AUTHOR CONTRIBUTIONS

ZZ and TV conceived the concept of the paper. TV wrote the draft. ZZ revised it. Both authors contributed to the article and approved the submitted version.

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Differential synaptic mechanism underlying the neuronal modulation of prefrontal cortex, amygdala, and hippocampus in response to chronic postsurgical pain with or without cognitive deficits in rats

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Chronic Postsurgical Pain (CPSP) is well recognized to impair cognition, particularly memory. Mounting evidence suggests anatomic and mechanistic overlap between pain and cognition on several levels. Interestingly, the drugs currently used for treating chronic pain, including opioids, gabapentin, and NMDAR (N-methyl-D-aspartate receptor) antagonists, are also known to impair cognition. So whether pain-related cognitive deficits have different synaptic mechanisms as those underlying pain remains to be elucidated. In this context, the synaptic transmission in the unsusceptible group (cognitively normal pain rats) was isolated from that in the susceptible group (cognitively compromised pain rats). It was revealed that nearly two-thirds of the CPSP rats suffered cognitive impairment. The whole-cell voltage-clamp recordings revealed that the neuronal excitability and synaptic transmission in the prefrontal cortex and amygdala neurons were enhanced in the unsusceptible group, while these parameters remained the same in the susceptible group. Moreover, the neuronal excitability and synaptic transmission in hippocampus neurons demonstrated the opposite trend. Correspondingly, the levels of synaptic transmission-related proteins demonstrated a tendency similar to that of the excitatory and inhibitory synaptic transmission. Furthermore, morphologically, the synapse ultrastructure varied in the postsynaptic density (PSD) between the CPSP rats with and without cognitive deficits. Together, these observations indicated that basal excitatory and inhibitory synaptic transmission changes were strikingly different between the CPSP rats with and without cognitive deficits.

KEYWORDS

chronic postsurgical pain, cognitive function, excitatory postsynaptic currents (EPSCs), inhibitory postsynaptic synaptic currents (IPSCs), postsynaptic density

Introduction

Chronic postsurgical pain (CPSP) affects nearly 10–50% of patients after surgery (Kehlet et al., 2006), impacting their quality of life negatively. CPSP is reported to impair cognition, including learning and memory (Cardoso-Cruz et al., 2013; Suto et al., 2014; Kehlet, 2018), which could be on account of pain using up a significant proportion of the limited cognitive resources of patients (Attal et al., 2014; Phelps et al., 2021). Conversely, cognitive functions may also influence the pain experienced by the patients (Bushnell et al., 2013; Matthewson et al., 2019; Howlin and Rooney, 2020). However, despite the universal agreement on the clinical significance of pain-related cognitive deficits, the underlying mechanisms have not been elucidated so far.

Previous studies have proposed the attentional cost hypothesis, brain plasticity, and structural changes in the brain as the possible mechanisms (Moriarty and Finn, 2014; Phelps et al., 2021). Depression, anxiety, sleep disturbances, fatigue, age, and generalized diffuse pain states might also be involved, according to certain studies (Mazza et al., 2018). However, in all of these previous studies, the animals that suffered chronic pain were placed in a single group without considering the difference between cognitively compromised pain animals and cognitively normal pain animals. Moreover, the drugs currently used for treating chronic pain, including gabapentin, opioids, and NMDAR (N-methyl-D-aspartate receptor) antagonists, are also indicated to impair memory (Kamboj et al., 2005; Morgan et al., 2014; Shem et al., 2018; Phelps et al., 2021). Therefore, it is of great importance to comprehensively investigate whether the pain-related cognitive deficits have different underlying mechanisms as those underlying the pain symptom.

As in the case of clinical surgical procedures, skin/muscle incision and retraction (SMIR) also leads to mechanical hypersensitivity, which is maintained mainly by supraspinal rather than purely spinal dysfunction (Flatters, 2008; Ying et al., 2014; Fitzcharles et al., 2021). In addition, the CPSP arising due to SMIR is not driven by neuronal damage because of a lack of either demyelination or injury in the saphenous nerve, further indicating the involvement of relevant central nervous system-related mechanisms (Flatters, 2008; Ying et al., 2014). Research has revealed that CPSP is the consequence of either nerve injury-induced neuropathic pain or the continuing inflammation (Kehlet et al., 2006). In this context, the SMIR model could be particularly relevant for the inflammation-induced pain that arises as a consequence of the elevated excitability of neurons in the central nervous system (central sensitization) (Kehlet et al., 2006). Moreover, studies associating synaptic transmission to pain and cognition are emerging (McCarberg and Peppin, 2019; Xiong et al., 2020). Collectively, these findings suggest that alterations in synaptic transmission might be associated with pain-related cognitive impairments. Anatomically, the medial prefrontal cortex (mPFC), central amygdala (CeA), and hippocampus, which are central to the modulation of cognition, are also implicated in the regulation of pain (Moriarty and Finn, 2014; McCarberg and Peppin, 2019; Phelps et al., 2021). Therefore, in the present study, the difference between the cognitively compromised pain animals and cognitively normal pain animals in terms of basal synaptic transmission from the mPFC, CeA, and hippocampal CA1 neurons was investigated.

Materials and methods

Experimental animals

Sprague-Dawley (SD) rats (male, weighing 200 ± 10 g each) procured from the Animal Center of Tongji Hospital were used in the experiments. All animals were housed under standard conditions (temperature 22°C–24°C, 12-h light/12-h dark photocycle) with *ad libitum* access to food and water. The experimental protocols were approved by the Animal Care and Use Committee, Tongji Hospital. All experiments were conducted by strictly following the instructions provided in the Guide for the Care and Use of the Laboratory Animals, National Institute of Health.

Behavior test

In order to assess whether the SMIR surgery evoked a significant mechanical hypersensitivity, mechanical sensitivity was measured. Subsequently, the open-field test was conducted to evaluate the locomotor activity (Liu et al., 2020). Finally, the Y-maze and novel object preference (NOP) tests were performed to estimate the degree of cognitive impairment induced by persistent pain hypersensitivity (Mathiasen and DiCamillo, 2010; Liu et al., 2020; Yuan et al., 2020). The cognitive behavior of each animal was monitored and analyzed simultaneously using an animal tracking system software (ANY-maze, Stoelting). After each trial, the test devices were cleaned to avoid interference due to odor. The results of the hierarchical clustering analysis in the Y-maze and the NOP tests revealed that the animals that suffered chronic pain could be classified into two groups: the unsusceptible group (comprising cognitively normal pain rats) and the susceptible group (comprising cognitively compromised pain rats).

Assessment of mechanical sensitivity

Mechanical sensitivity was assessed based on the paw withdrawal threshold (PWT) test using grade-strength von

Frey monofilaments (1.0–15 g) as described in a previous report (Li et al., 2016, 2017). Prior to the test, the rats were placed inside separate plastic chambers over mesh platforms and acclimated for over 30 min to achieve immobility. Subsequently, according to the up–down paradigm, beginning with a quantity of 1.0 g, filaments of sequentially increasing stiffness were applied vertically to the mid-plantar surface of the right hind paw of each rat until the filament bent. A positive response was defined as brisk withdrawal or licking the paw. A minimum of 5 min interval was maintained between adjacent tests.

Open field test

After 1 h of acclimatization to the testing room, each rat was placed in the center of a black open-field chamber (size 100 × 100 × 40 cm), where it was allowed to move spontaneously for 5 min. The total distance traveled by the rat was recorded and used for measuring the rat's locomotor activity (Liu et al., 2020).

Novel object preference test

The NOP test was conducted to evaluate the non-spatial visual learning memory. The test was conducted within a black open field with two stages. At the training stage, after accommodation without objects, the rat was allowed for 5 min to freely explore the two exact things pasted on the central symmetrical positions of the field. Two hours later, the rat was placed in the same region again and allowed to free exploration for 5 min once again, this time with one of the objects being replaced by a novel, unfamiliar object (test session). The time spent exploring the familiar object (F), and the novel object (N) was recorded. The recognition index, reflecting the memory function, was calculated using the formula (N-F)/(N + F)*100% (Mathiasen and DiCamillo, 2010; Xiong et al., 2020).

Y-maze test

The Y-maze test was conducted to evaluate the spatial orientation learning memory ability of the rats. The Y-maze comprised three identical arms that converged to an equal angle. The randomly named start arm (animal entry) and the other arm remained open always. Initially, the new arm was blocked, while the other two arms were available to the rat for free exploration for 5 min. After 2 h, all the arms were opened, allowing the rat free access to all three arms for 5 min. The entries into the new arm and the time spent in the new arm reflected the spatial recognition memory (learned behavior) (Liu et al., 2020).

Skin/muscle incision and retraction model

The SMIR surgery of rats was performed as described in previous reports (Flatters, 2008; Liu et al., 2021). The rats were anesthetized using pentobarbital sodium (50 mg/kg, intraperitoneally) and placed on their backs, followed by shaving the right medial thigh region and sterilizing it using alcohol. A 1.5–2 cm incision (approximately 4 mm medial to the saphenous vein) was performed in the skin of the medial thigh to expose the muscle of the thigh. Next, a 1 cm incision (approximately 3 mm proximal to the saphenous nerve) was performed in the superficial muscle layer. Subsequently, the external muscle was parted via a blunt dissection to insert a retractor into the incision site. The skin and superficial muscle of the thigh were retracted by 2 cm to expose the fascia underlying the adductor muscles for 1 h. Sham rats underwent the same procedure without skin/muscle retraction. The main objective of the SMIR model was to mimic the clinical scenario, in which persistent postoperative pain was evoked by SMIR rather than by neuronal damage.

Electrophysiology of brain slices

As described previously (Li et al., 2016, 2017), whole-cell recordings from the anterior cingulate cortex (the dorsal component of mPFC), hippocampal CA1, and CeA neurons were obtained and analyzed to reveal the alterations in synaptic transmission. After the behavioral tests (**Figure 1A**), rats in each group were anesthetized using 2% sodium pentobarbital (30 mg/kg, intraperitoneal injection), and their brain tissues were dissected and placed into a cutting solution at 4°C. The dissection solution comprised the following (in mM): glucose, 10; sucrose, 213; KCl, 3; $NaHCO_3$, 26; NaH_2PO_4, 1.2; $CaCl_2$, 0.5; $MgCl_2$, 5. After a recovery period of at least 1 h at room temperature in oxygenated artificial cerebrospinal fluid (ACSF), an individual slice was transferred to a submersion-recording chamber that was being continuously perfused with ACSF at a flow rate of 2 mL/min. The ACSF comprised the following (in mM): NaCl, 125; $NaHCO_3$, 26; KCl, 5; NaH_2PO_4, 1.2; $CaCl_2$, 2.6; $MgCl_2$, 1.3; glucose, 10. Only one neuron was recorded from each slice. In one animal, two neurons were recorded from mPFC, CeA, and hippocampal CA1 each.

The whole-cell recordings from visually identified ACC, CeA, and hippocampal CA1 excitatory pyramidal neurons were obtained using an Axonpatch 700B amplifier (Molecular Devices, United States). The recordings were digitized using a Digidata 1500B digital converter (Molecular Devices, United States). The spontaneous and miniature excitatory postsynaptic currents (sEPSCs/mEPSCs) and the spontaneous and miniature inhibitory postsynaptic

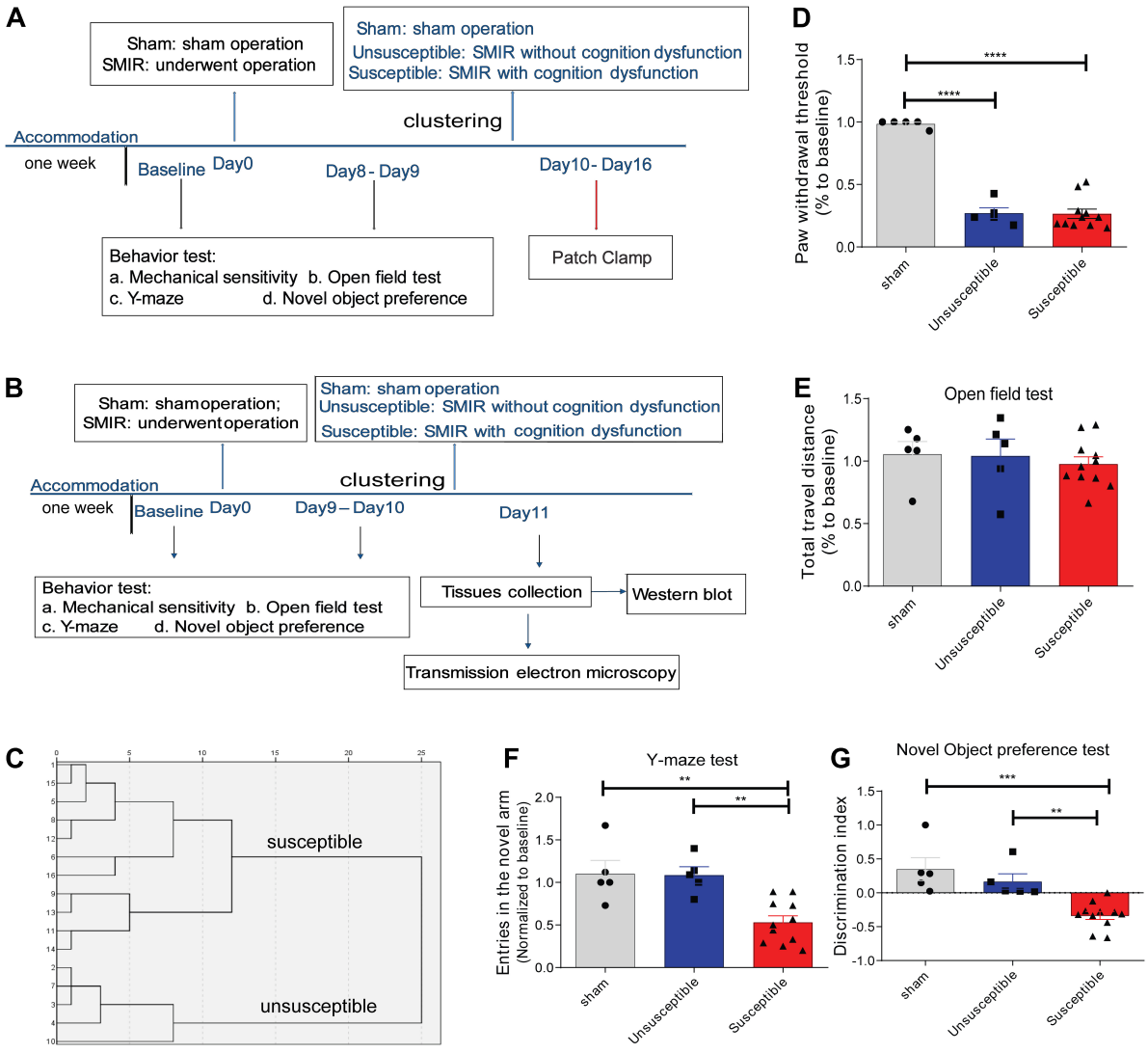

FIGURE 1
SMIR-induced significant mechanical hypersensitivity and cognitive impairment in rats. **(A,B)** Schematic illustration of the design of the complete experiment. **(C)** Dendrogram of the hierarchical clustering analysis. The rats who underwent the SMIR surgery were divided into the susceptible group (CPSP rats with cognitive dysfunction, $n = 11$) and the unsusceptible group (CPSP rats without cognitive dysfunction, $n = 5$) according to the hierarchical clustering analysis of the data from the Y-maze test and the novel object preference test. **(D–G)** Behavioral test after SMIR surgery, including **(D)** mechanical sensitivity ($F = 90.07$, $p < 0.0001$), **(E)** the open field test ($F = 0.28$, $p = 0.76$), **(F)** the Y-maze ($F = 11.40$, $p = 0.0006$), **(G)** the novel object preference test ($F = 14.21$, $p = 0.0002$). Results are expressed as mean ± SEM; Tukey's *post-hoc* tests; $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

currents (sIPSCs/mIPSCs) were recorded at −70 mV. The WPI recording pipettes (4–8 MΩ, United States) were filled with an intracellular solution comprising the following (in mM): potassium-gluconate, 122; NaCl, 5; $MgCl_2$, 2; $CaCl_2$, 0.3; HEPES, 10; EGTA, 5; Mg-ATP, 4; Na_3-GTP, 0.3 (pH 7.30–7.35 and 300 mOsm/kg). The recording was carried out using microelectrodes filled with K + gluconate (for action potentials and sEPSCs/mEPSCs recordings) or KCl (for sIPSCs/mIPSCs determinations) (Sceniak et al., 2016). The KCl solution consisted of (in mM): 100 KCl, 40 HEPES, 0.2 EGTA, 5 $MgCl_2$, 2 Mg-ATP, 0.3 Na_3-GTP, 10 phosphocreatine. The mEPSCs were recorded with picrotoxin (100 μM) and tetrodotoxin (TTX) (1 μM) added to the ACSF, and the mIPSCs were pharmacologically isolated using CNQX (10 μM), AP-5 (100 μM), and TTX (1 μM). The sEPSCs/sIPSCs were recorded without TTX added to the ACSF. The excitatory pyramidal neurons and the inhibitory interneurons in different regions were identified by accommodating responses to a sustained depolarizing intracellular current stimulation. Regular spiking neurons with a firing rate of < 5 Hz were believed to correspond to the excitatory pyramidal neurons, while the fast-spiking neurons with a firing rate of > 10 Hz

and shorter duration waveforms were believed to correspond to the inhibitory interneurons (Laviolette and Grace, 2006; Koyanagi et al., 2021). We only recorded the excitatory neurons. The number of action potentials (APs) evoked upon the intracellular current injections of increasing magnitude (from 0 to 300 pA, with steps of 50 pA; duration 500 ms) was counted to determine the passive membrane property and the single AP characteristics without using any synaptic blocker. High seal (GΩ) and low series (30 MΩ) resistances were monitored throughout the experiment (using the membrane test function) to ensure a high-quality recording. A fixed length of traces (5 min) was analyzed to obtain the frequency and amplitude of sEPSCs/sIPSCs or mEPSCs/mIPSCs with pCLAMP10.7.

Western blotting

Immediately after the rats were euthanized (**Figure 1B**), their mPFC, CeA, and hippocampal CA1 were retrieved immediately through dissection and stored at −80°C until analysis (Li et al., 2016, 2017). The tissues were homogenized and lysed using a radio-immuno-precipitation assay buffer (AR0102, Boster) and then subjected to the measurement of protein concentration using the bicinchoninic acid kit (AR1189, Boster). The lysates (20 μg each) were separated on 10% SDS-PAGE gels, and the separated protein bands were transferred electrophoretically to a polyvinylidene fluoride (PVDF) membrane. Immunoreactivity was determined based on enhanced chemiluminescence, and the signals were detected using a Bio-Rad ChemiDoc system (Bio-Rad Laboratories, China).

The following antibodies were used: anti-GAPDH (1:5,000, BM1623, Boster); anti-GluA1 (1:1,000, A1826, ABclonal); anti-mGluR1 (1:1,000, abx112750, Abbexa); anti-mGluR5 (1:1,000, A3758, ABclonal); anti-GluN2B (1:1,000, abx23583, Abbexa); anti-PSD-95 (1:1,000, abx236850, Abbexa); anti-α5 GABA (1:1,000, ab259880, Abcam). The secondary antibodies used were goat anti-rabbit IgG (1:10,000; Boster) and goat anti-mouse IgG (1: 10,000, Boster). The immunosignals were quantified using densitometry and were expressed relative to the GAPDH signals and normalized to the control for data analysis.

Electron microscopy

The ultrastructures of the synapses in the mPFC, CeA, and hippocampal CA1 regions of rats from the sham, unsusceptible, and susceptible groups were examined using a transmission electron microscope (TEM) (**Figure 1B**). In brief, the respective regions were split and fixed successively in 2.5% glutaraldehyde (cold) and 1% OsO_4 at 4°C (Mannaioni et al., 2001). After three washes with PBS, the specimens were dehydrated in a series of ethanol solutions. Subsequently, the samples were infiltrated and embedded in pure LR-White resin. Ten ultrathin sections (50 nm) were prepared from each region and then stained with uranyl acetate and lead citrate. The stained sections were observed under a JEM-1230 transmission electron microscope operating at 120 kV. Asymmetric (glutamatergic) synapses were characterized by thick electron-dense post-synaptic specializations while symmetric (GABAergic) synapses had thin post-synaptic specializations (Fitzgerald et al., 2019). Only the excitatory (asymmetric) synapses were examined.

Statistical analysis

All result data were expressed as mean ± SEM and subjected to the analysis of significant differences between the mean values using a Tukey's test after the normality distribution assessment that was evaluated using the Kolmogorov-Smirnov test. Hierarchical cluster analysis was performed according to a previous study conducted by our research group (Li et al., 2021). The rats that underwent the SMIR surgery were divided into the susceptible group (cognitively compromised pain rats) and the unsusceptible group (cognitively normal pain rats) based on the Ward method and Euclidean distance square as distance measurement using the hierarchical cluster analysis of the data from the NOP and Y-maze tests. The significance was tested using two-tailed tests, and a p-value of less than 0.05 was considered statistically significant. All graphs and statistical analyses were conducted using GraphPad Prism 8.0 and Adobe Illustrator Artwork 14.0.

Results

Skin/muscle incision and retraction-evoked significant mechanical hypersensitivity and lower cognition in rats

According to the results of a previous study, the SMIR-evoked mechanical hypersensitivity appeared by the postoperative Day 3, with the maximum level appearing between the postoperative Day 10 and Day 13, and persisted until the postoperative Day 22 (Flatters, 2008). Therefore, in the present study, the PWT was assessed on Day 8 or Day 9, and the cognitive behavior was evaluated on Day 9 or Day 10 after surgery (**Figures 1A,B**). According to our hierarchical clustering analysis of the cognitive behavior evaluation data (**Figure 1C**) from the NOP and Y-maze tests, the rats who underwent the SMIR surgery were divided into

the susceptible group (n = 11) and the unsusceptible group (n = 5). As depicted in **Figure 1D**, the rats who underwent the SMIR surgery exhibited significantly decreased PWT. In the cognitive behavior test, the three groups exhibited no significant differences in terms of the total distance traveled (**Figure 1E**), suggesting no damage to motor function upon the SMIR surgery. In comparison to the sham and unsusceptible group rats, the rats in the susceptible group presented fewer entries into the new arm and exhibited a discrimination ability between the novel and the familiar object (**Figures 1F,G**), indicating poorer cognitive performance. Meanwhile, the sham group and the unsusceptible group rats exhibited no significant difference in this regard. These results verified that SMIR surgery leads to significant mechanical hypersensitivity and cognitive deficits in rats.

Neuronal excitability alterations in the medial prefrontal cortex, central amygdala, and hippocampus pyramidal neurons after the skin/muscle incision and retraction surgery

The spiking frequency in the anterior cingulate cortex (the dorsal component of mPFC), CeA, and hippocampal CA1 excitatory neurons was determined to assess the intrinsic neuronal excitability after the SMIR surgery in all three groups (**Figure 2**). The number of depolarization-induced spikes generated upon intracellular injections of depolarizing current pulses was significantly different among the three groups. As depicted in the figure, the number of spikes in the mPFC and CeA neurons after SMIR was higher in the unsusceptible group rats compared to the sham group (**Figures 2A,B**), while the number of spikes in the susceptible group rats was lower than that in the unsusceptible group rats. On the contrary, neuronal excitability in the hippocampal CA1 neurons was decreased in the unsusceptible group rats and not in the susceptible group rats (**Figure 2C**). These results demonstrated pronounced differences in the intrinsic neuronal excitability in mPFC, CeA, and hippocampus pyramidal neurons between the CPSP rats with and without cognitive deficits.

There were apparent discrepancies in the synaptic transmissions from the medial prefrontal cortex and central amygdala pyramidal neurons between the chronic postsurgical pain rats with and without cognitive deficits

In order to evaluate whether alterations in synaptic transmission participate in the development of the cognitive deficits induced by CPSP, the excitatory synaptic transmission in the anterior cingulate cortex (the dorsal component of mPFC) and CeA pyramidal neurons in brain slices were recorded. The electrophysiology data suggested that compared to the sham group, the unsusceptible group exhibited significantly enhanced frequency of sEPSCs/mEPSCs in mPFC (**Figures 3A–D**) and CeA (**Figures 4A–D**) excitatory neurons. Interestingly, compared to the unsusceptible group, the susceptible group exhibited decreased synaptic transmission. No noticeable difference could be observed in the amplitudes of the excited sEPSCs/mEPSCs.

Moreover, since Gamma-aminobutyric acid (GABA) systems play crucial roles in learning and memory (He et al., 2019), GABA-driven inhibitory synaptic transmission was also recorded in the present study. Similarly, the frequency of sIPSCs/mIPSCs in the mPFC and CeA neurons exhibited the same tendency as that of the sEPSCs/mEPSCs from the three groups. As depicted in **Figures 3E–H**, **4E–H**, the frequency of sIPSCs/mIPSCs in the mPFC and CeA neurons from the unsusceptible group was elevated. In addition, the amplitude of the mIPSCs of the mPFC and CeA neurons in susceptible group rats was lower than that in the unsusceptible group rats. These results suggested discrepancies in both excitatory and inhibitory synaptic transmission between the CPSP rats with and without cognitive deficits in the mPFC and CeA neurons.

Synaptic transmissions in the hippocampal CA1 pyramidal neurons varied markedly between the chronic postsurgical pain rats with and without cognitive deficits

Since the hippocampus plays a significant role in pain and cognition, the synaptic transmissions in the hippocampal CA1 neurons of the rats from the three groups were also recorded in the present study. As depicted in **Figure 5**, compared to the sham group, the unsusceptible rats exhibited a significant decline in the frequencies of sEPSCs/mEPSCs and sIPSCs/mIPSCs in the hippocampal CA1 neurons. Meanwhile, compared to the unsusceptible group, the susceptible group exhibited a significant elevation in the frequencies of sEPSCs/mEPSCs and sIPSCs/mIPSCs in the hippocampal CA1 neurons. Moreover, the amplitudes of the sIPSCs in the unsusceptible group and those of mIPSCs in the susceptible group were lower than those in the sham group (**Figures 5F,H**). These results revealed that the excitatory and inhibitory synaptic transmissions in the CA1 neurons differed markedly between the CPSP rats with or without cognitive deficits.

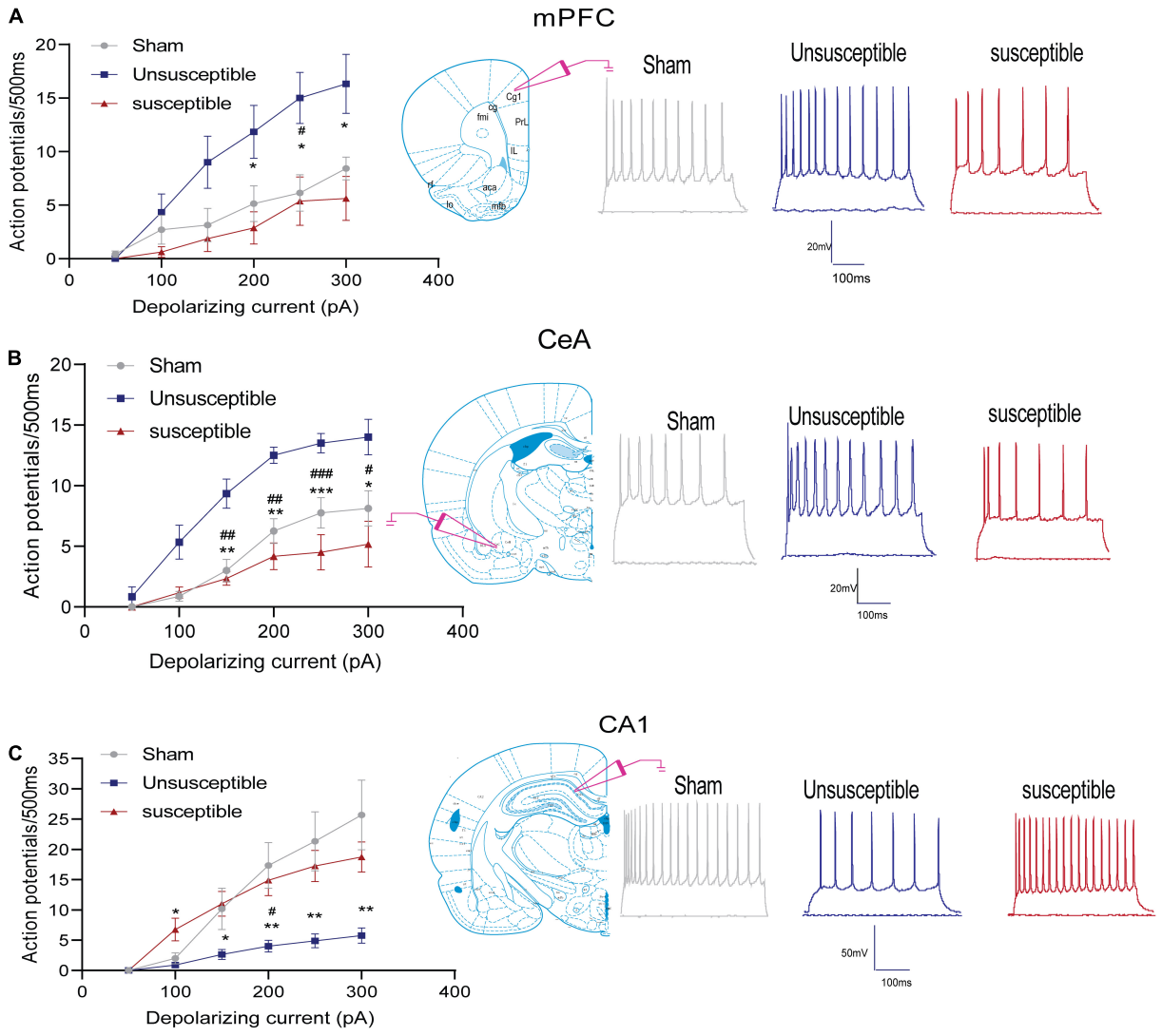

FIGURE 2
Neuronal excitability in the mPFC, hippocampus, and CeA pyramidal neurons after the SMIR surgery. Graphs present the mean frequency of AP (action potential) fired in response to the 500 ms current injection ranging from 0 to 300 pA (left). Traces present the potential firings evoked by the 500 ms depolarizing current steps of 300 pA (right). **(A)** Quantification of the AP firing frequencies in the anterior cingulate cortex of the mPFC neurons; $N = 5$ rats/group, $F = 30.64$, $p < 0.0001$. **(B)** Quantification of the AP firing frequencies in the CeA neurons; $N = 5$ rats/group, $F = 18.16$, $p < 0.0001$. **(C)** Quantification of the AP firing frequencies in the hippocampal CA1 neurons; $N = 5$ rats/group, $F = 8.27$, $p = 0.0026$. Statistical significance was determined by two-way ANOVA followed by Bonferroni's *post-hoc* test. Sham vs. Unsusceptible, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; Unsusceptible vs. Susceptible, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Changes in the protein levels of mGluR1, mGluR5, GluN2B, GluA1, PSD-95, and α5-gamma-aminobutyric acid in medial prefrontal cortex, central amygdala, and hippocampus after the skin/muscle incision and retraction surgery

Considering the discrepancies in the synaptic transmissions from the mPFC, hippocampus, and CeA regions of the mouse brain among the three groups, the levels of synaptic transmission-related proteins were evaluated next. GluA1, which is one of the subunits of AMPAR (a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor), and NMDAR mediate most of the excitatory synaptic transmissions, while GABAR mediates most of the inhibitory synaptic transmissions. A dysregulation in the levels of PSD95 and GABA reportedly contributes to pain and memory impairment (Perez-Sanchez et al., 2017; Coley and Gao, 2019; Kumari et al., 2020), while reduced PSD95 levels are predictive of cognitive deficits (Whitfield et al., 2014). In view of the role of

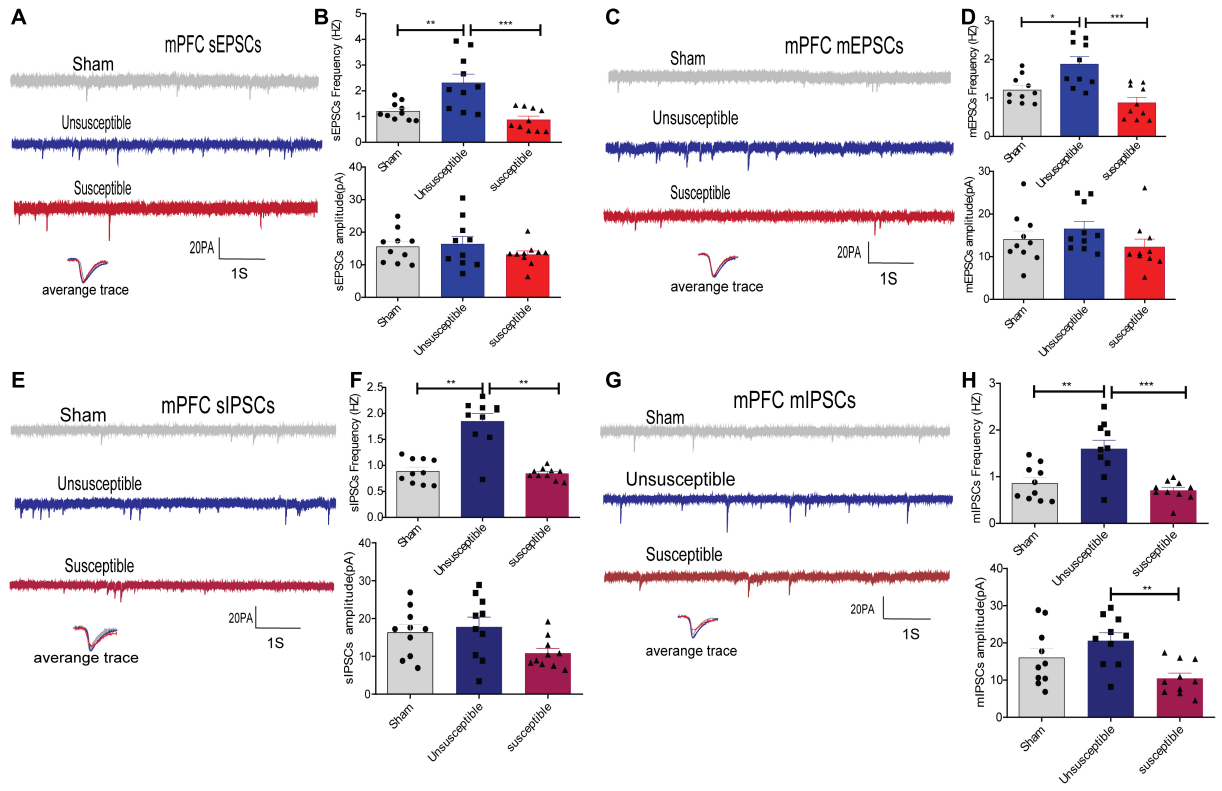

FIGURE 3
Apparent discrepancies in the synaptic transmissions in the anterior cingulate cortex of mPFC pyramidal neurons between the CPSP rats with and without cognitive deficits. **(A,C)** A typical time-course with traces of sEPSCs/mEPSCs in the individual slices from three groups of rats (above) and the individual traces (average) of sEPSCs/mEPSCs obtained from the corresponding recordings (bottom). Calibration: 20 pA. **(B,D)** Bar graphs presenting the frequencies and amplitudes of the sEPSCs/mEPSCs. sEPSCs (frequency: one-way ANOVA, $F = 12.29$, $P = 0.0002$; amplitude: one-way ANOVA, $F = 0.94$, $P = 0.40$), mEPSCs (frequency: one-way ANOVA, $F = 11.98$, $P = 0.0002$; amplitude: one-way ANOVA, $F = 1.38$, $P = 0.27$). **(E,G)** Representative sIPSCs/mIPSCs in the pyramidal neurons recorded from the three groups of rats (above) and the individual traces (average) of sIPSCs/mIPSCs obtained from the corresponding recordings (bottom). Calibration: 20 pA. **(F,H)** Bar graphs presenting the frequencies and amplitudes of sIPSCs/mIPSCs. sIPSCs (frequency: Kruskal-Wallis test, $P = 0.0009$; amplitude: one-way ANOVA, $F = 3.21$, $P = 0.06$), mIPSCs (frequency: one-way ANOVA, $F = 12.86$, $P = 0.0001$; amplitude: one-way ANOVA, $F = 6.16$, $P = 0.006$). Results are expressed as mean ± SEM; $n = 10$ neurons from 5 rats/group; Tukey's *post-hoc* tests; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (compared to sham and unsusceptible groups, respectively).

Group I mGluRs (mGluR1 and mGluR5) in pain (Zhou et al., 2017) and cognition (Liu et al., 2012), the protein levels of mGluR1 and mGluR5 were also determined in the present study.

The western blotting results revealed a significant increase in the post-SMIR protein levels of mGluR1, GluN2B, GluA1, PSD-95, and α5GABA in mPFC and CeA in the unsusceptible group compared to the sham group (**Figures 6A,B**), which was not observed in the susceptible group. Meanwhile, the levels of mGluR5 in mPFC in the unsusceptible group and susceptible group were lower than those in the sham group. Similarly, in the unsusceptible group, the protein levels of mGluR1, mGluR5, GluN2B, GluA1, PSD-95, and α5GABA from the hippocampus were decreased, while the same was not true for the susceptible group (**Figure 6C**). Collectively, these results indicated that the levels of synaptic neurotransmitter receptors differed significantly between the CPSP rats with cognitive deficits and the CPSP rats without cognitive deficits.

The ultrastructures of the synapses from the medial prefrontal cortex, hippocampal CA1, and central amygdala regions varied in their postsynaptic density after the skin/muscle incision and retraction surgery

In order to establish that the excitatory and inhibitory synaptic transmission disorders were due to the presynaptic or postsynaptic deficits, the synaptic vesicles and postsynaptic density (PSD) in the anterior cingulate cortex (the dorsal

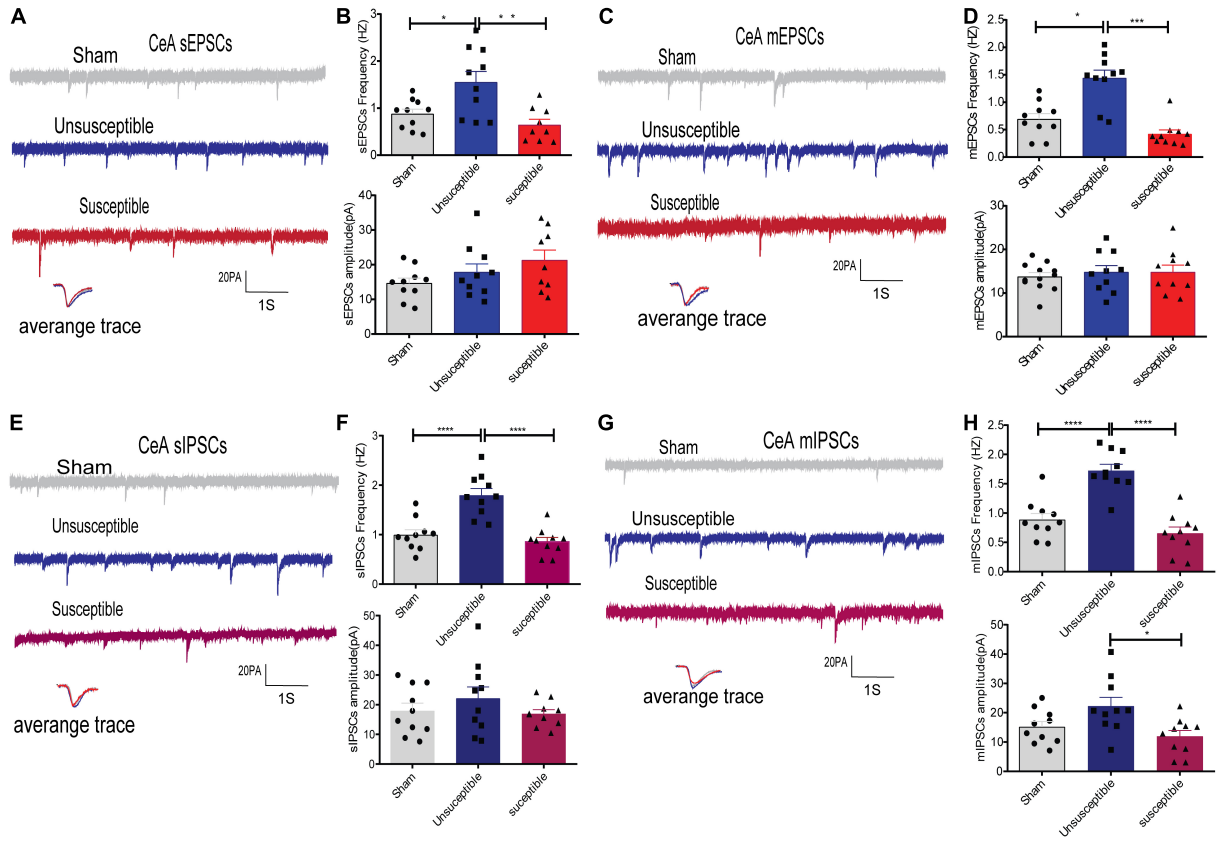

FIGURE 4
Evident differences in the synaptic transmission from the CeA pyramidal neurons between the cognitively normal and cognitively compromised pain rats. **(A,C)** Sample recordings of sEPSCs/mEPSCs in the individual slices from the three groups (above) and the individual traces (average) of sEPSCs/mEPSCs obtained from the corresponding recordings (bottom). Calibration: 20 pA. **(B,D)** Bar graphs presenting the frequencies and amplitudes of sEPSCs/mEPSCs. sEPSCs (frequency: one-way ANOVA, $F = 8.46$, $P = 0.002$; amplitude: one-way ANOVA, $F = 2.02$, $P = 0.15$), mEPSCs (frequency: Kruskal-Wallis test, $P = 0.0002$; amplitude: one-way ANOVA, $F = 0.24$, $P = 0.79$). **(E,G)** Representative sIPSCs/mIPSCs in the pyramidal neurons from the three groups (above) and the individual traces (average) of sIPSCs/mIPSCs obtained from the corresponding recordings (bottom). Calibration: 20 pA. **(F,H)** Bar graphs presenting the frequencies and amplitudes of the sIPSCs/mIPSCs. sIPSCs (frequency: one-way ANOVA, $F = 21.48$, $P < 0.0001$; amplitude: one-way ANOVA, $F = 0.98$, $P = 0.39$), mIPSCs (frequency: one-way ANOVA, $F = 28.23$, $P < 0.0001$; amplitude: one-way ANOVA, $F = 5.13$, $P = 0.01$). Results are expressed as mean ± SEM; $n = 10$ neurons from 5 rats/group; Tukey's *post-hoc* tests; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.001$ (compared to sham and unsusceptible groups, respectively).

component of mPFC), hippocampus, and CeA specimens from the three groups were investigated (Figures 7A–C). The electron microscopy results presented in Figures 7D–F revealed no significant difference in the number of synapses among the groups at the presynaptic level. However, at the postsynaptic level (Figures 7G,H), the length of PSD in the mPFC and CeA specimens from the susceptible group rats was lower than the corresponding value in the sham and unsusceptible group rats. In addition, the length of PSD in the hippocampus of rats from the susceptible group was reduced compared to the unsusceptible rats (Figure 7I). However, compared to the sham group, the unsusceptible group exhibited an elevated length of PSD in the hippocampus region of rats after SMIR (Figure 7I). No noticeable difference could be observed in the width of PSD in the three groups (Figures 7J–L). Collectively, these results suggested significant discrepancies in the synapse ultrastructure that were closely related to CPSP and the associated cognitive dysfunction in rats.

Discussion

Substantial evidence suggests the detrimental effect of chronic pain on cognition (Cowen et al., 2018; Matthewson et al., 2019; Phelps et al., 2021). Nonetheless, whether cognitively compromised animals with pain have the exact same synaptic mechanisms as those in the cognitively normal animals with pain remains to be elucidated so far. The present study revealed that nearly two-thirds of the CPSP rats suffered cognitive impairment, which is consistent with the findings of previous clinical studies (Dick and Rashiq, 2007;

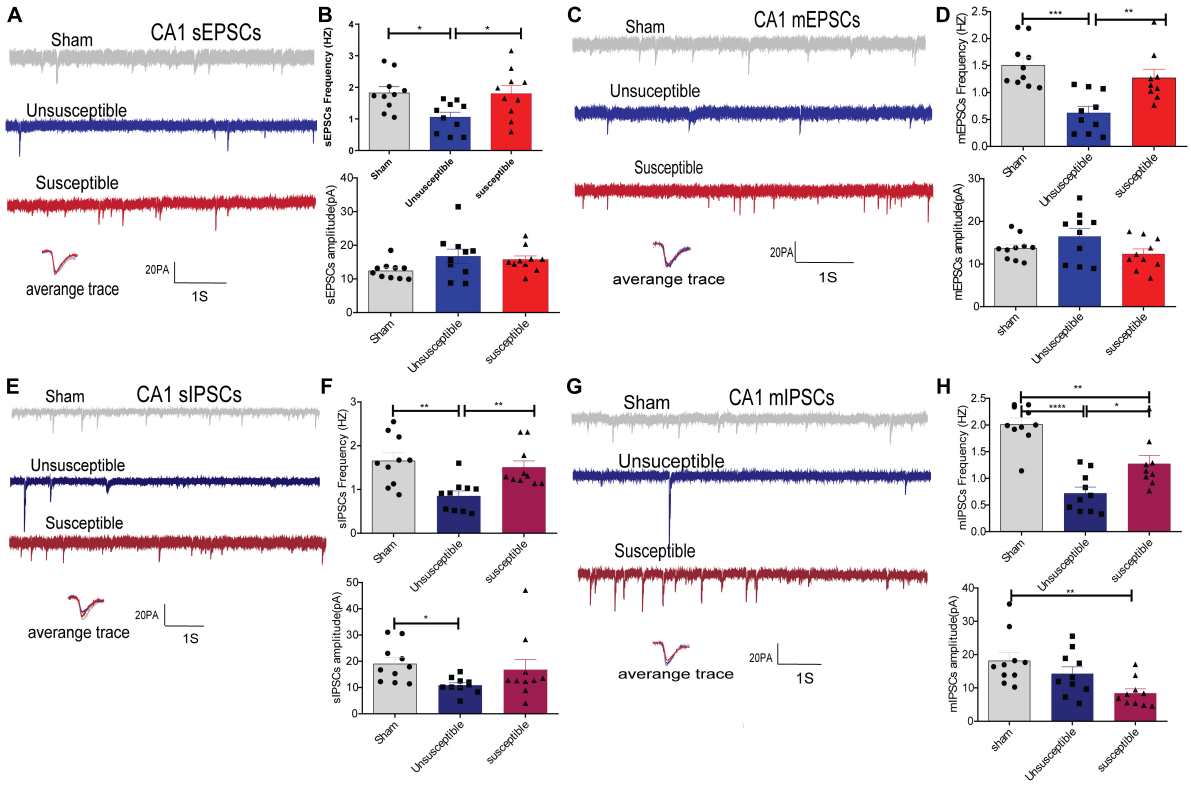

FIGURE 5
CPSP rats with and without cognitive deficits exhibited marked differences in the synaptic transmissions in hippocampal CA1 pyramidal neurons. **(A,C)** A typical time-course with traces showing a continuous recording of sEPSCs/mEPSCs taken from groups after SMIR surgery. Calibration: 20 pA. **(B,D)** Bar graphs present the frequencies and amplitude of sEPSCs/mEPSCs. sEPSCs (frequency: one-way ANOVA, $F = 4.94$, $P = 0.01$; amplitude: one-way ANOVA, $F = 2.31$, $P = 0.12$), mEPSCs (frequency: one-way ANOVA, $F = 11.81$, $P = 0.0002$; amplitude: one-way ANOVA, $F = 0.41$, $P = 0.11$). **(E,G)** Representative sIPSCs/mIPSCs recorded in the CA1 pyramidal neurons from the three groups (above) and the individual traces (average) of sIPSCs/mIPSCs obtained from the corresponding recordings (bottom). Calibration: 20 pA. **(F,H)** Bar graphs presented the frequencies and amplitudes of sIPSCs/mIPSCs. sIPSCs (frequency: Kruskal-Wallis test, $P = 0.0002$; amplitude: Kruskal-Wallis test, $P = 0.02$), mIPSCs (frequency: one-way ANOVA, $F = 26.28$, $P < 0.0001$; amplitude: Kruskal-Wallis test, $P = 0.004$). Results are expressed as mean ± SEM; $n = 10$ cells from 5 rats/group; Tukey's *post-hoc* tests; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.001$ (compared to sham and unsusceptible groups, respectively).

Berryman et al., 2013). The most exciting finding of the present study was that spontaneous synaptic transmissions differed significantly between the CPSP rats with and without lower cognition. This finding was further corroborated by the variations observed in the levels of synaptic transmission-related proteins and the synapse ultrastructure. Overall, using a combination of different approaches, it was identified that CPSP rats with lower cognition exhibited strikingly different synaptic transmission compared to the CPSP rats without lower cognition. This finding would further advance the current understanding of the relationship between pain and pain-related cognition.

The proteins mGluR1 and mGluR5 are implicated in pain and cognition. These proteins play diverse roles in increasing neuronal excitability by modulating various ion channels and the associated proteins in the mPFC, CeA, and hippocampal CA1 pyramidal neurons (Mannaioni et al., 2001). For instance, in CA1 pyramidal cells, mGluR1 activation reportedly led to somatic calcium transients and direct neuronal depolarization, while mGluR5 activation inhibited the gradual after-hyperpolarization potential potassium currents and enhanced the NMDA receptor currents (Niswender and Conn, 2010). The accumulation of the GluN2B-NMDA receptors at PSD was anticipated to intensify a range of Ca^{2+}–sensitive signaling cascades, which would contribute to the increased neuronal excitability and enhanced responsiveness to peripheral stimuli (Qiu et al., 2011). The present study revealed that neuronal excitability was increased in the mPFC and CeA neurons in cognitively normal rats with pain. This was consistent with the previous studies that reported increased excitability in the mPFC pyramidal cells after peripheral inflammation (Ong et al., 2019) and in the CeA pyramidal neurons in acute arthritis and neuropathic pain models (Jiang et al., 2014; Thompson and Neugebauer, 2019). Accordingly, a significant increase in the levels of mGluR1, mGluR5, and NMDAR-subunit N2B proteins was observed in the CeA of

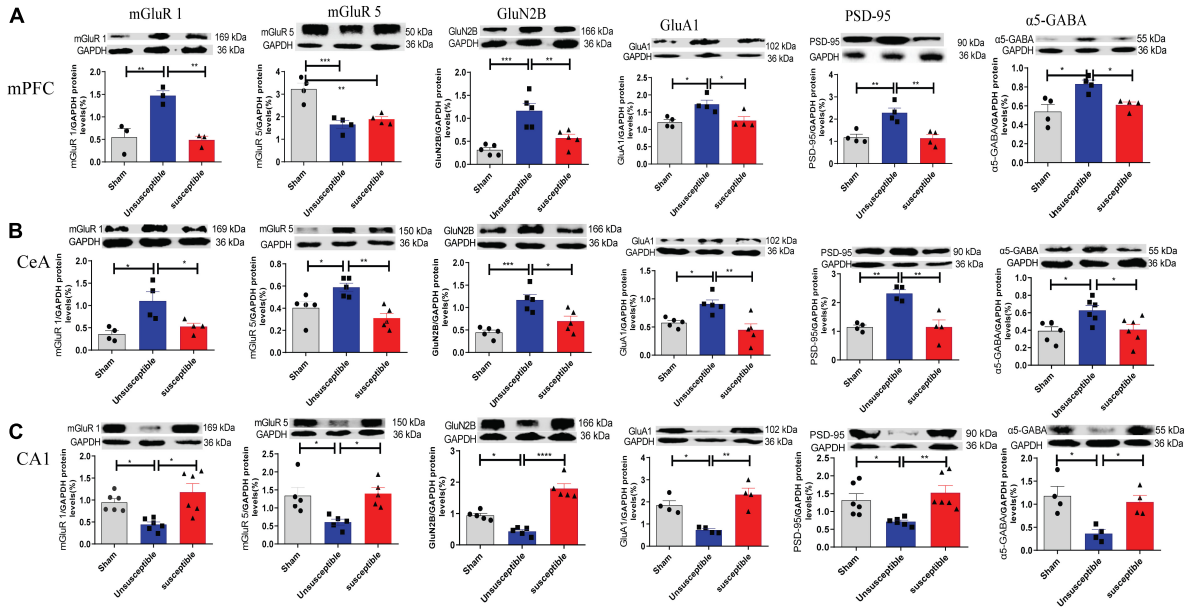

FIGURE 6
Changes in the levels of mGluR1, mGluR5, GluN2B, GluA1, PSD-95, and α5-GABA in mPFC, CeA, and hippocampus, 11 days after the SMIR surgery. **(A)** The representative Western blots and quantification of synaptic transmission-related proteins in mPFC. one-way ANOVA, mGluR1 ($F = 16.24$, $P = 0.004$), mGluR5 ($F = 19.42$, $P = 0.0005$), GluN2B ($F = 16.28$, $P = 0.0004$), GluA1 ($F = 7.54$, $P = 0.02$), PSD-95 ($F = 13.36$, $P = 0.002$), and α5-GABA ($F = 8.31$, $P = 0.009$). **(B)** Changes in the levels of synaptic transmission-related proteins in CeA. one-way ANOVA, mGluR1 ($F = 8.23$, $P = 0.009$), mGluR5 ($F = 9.72$, $P = 0.003$), GluN2B ($F = 13.58$, $P = 0.0008$), GluA1 ($F = 9.05$, $P = 0.004$), PSD-95 ($F = 16.19$, $P = 0.001$), and α5-GABA ($F = 5.74$, $P = 0.01$). **(C)** Changes in the expression levels of synaptic transmission-related proteins in the hippocampus. one-way ANOVA, mGluR1 ($F = 9.03$, $P = 0.002$), mGluR5 ($F = 6.85$, $P = 0.01$), GluN2B ($F = 44.96$, $P < 0.0001$), GluA1 ($F = 15.50$, $P = 0.001$), PSD-95 ($F = 7.17$, $P = 0.006$), and α5-GABA ($F = 8.26$, $P = 0.009$). Results are expressed as mean ± SEM; Tukey's *post-hoc* tests; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$; $n = 3$–6/group.

the cognitively normal pain rats. Furthermore, the neuronal excitability in the hippocampal CA1 neurons of cognitively normal pain animals was decreased. These results were consistent with the previous reports that reported prolonged decreases in the cellular activity of CA1 pyramidal neurons in response to painful stimuli (McEwen, 2001) and the association of the increased neuronal excitability in the CA1 pyramidal neurons with cognition function (Wang W. et al., 2020). As expected, the levels of mGluR1, mGluR5, and NMDAR-subunit N2B proteins in hippocampal CA1 were significantly reduced in the cognitively normal pain animals.

So far, it was clear that the alterations in the neuronal excitability due to dynamic changes in the levels of mGluR1, mGluR5, and NMDAR-subunit N2B proteins in the mPFC, CeA, and hippocampal CA1 pyramidal neurons were involved in the development of CPSP and the associated lower cognition. However, the causal relationship between neuronal excitability and mechanical hypersensitivity and the lower cognition of animals remained unclear. It is noteworthy that additional evidence concurs the deactivation of mPFC during chronic pain (Wang et al., 2015). These discrepancies could be explained by the differences observed in the record regain, animal strains, pain models, and points in time. In addition, Ji et al. (2010) reported that modulation of CeA does not affect arthritis pain-related dysfunction of decision-making. Again, this discrepancy could be partially explained by the difference in the animal models used or the different aspects of cognitive function.

PSD-95 is a major scaffolding protein in the PSD of excitatory synapses. PSD-95 plays a critical role in pain and cognition-related glutamatergic synaptic transmission by interacting with and functionally influencing the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and NMDA receptors (Qiu et al., 2011; Ong et al., 2019). For instance, PSD-95 deficiency is reported to disrupt the synaptic NMDAR/AMPAR current and the levels of associated proteins in the hippocampus and mPFC, thereby inducing learning and working memory deficits (Chen et al., 2015; Coley and Gao, 2019). In addition, an increase in the PSD-95 protein levels reflects an enhanced number of synapses (Seo et al., 2014), while aberrant regulation of GluA1 functions or dynamics is directly associated with cognitive impairment (Wang et al., 2017). Therefore, reduced PSD-95 or GluA1 levels are routinely used for predicting cognitive deficits (Whitfield et al., 2014; Ding et al., 2016). Interestingly, in the present study, the PSD-95 or GluA1 protein levels in the hippocampus were lower than in the cognitively normal pain animals compared to cognitively compromised pain animals, which was consistent with previous

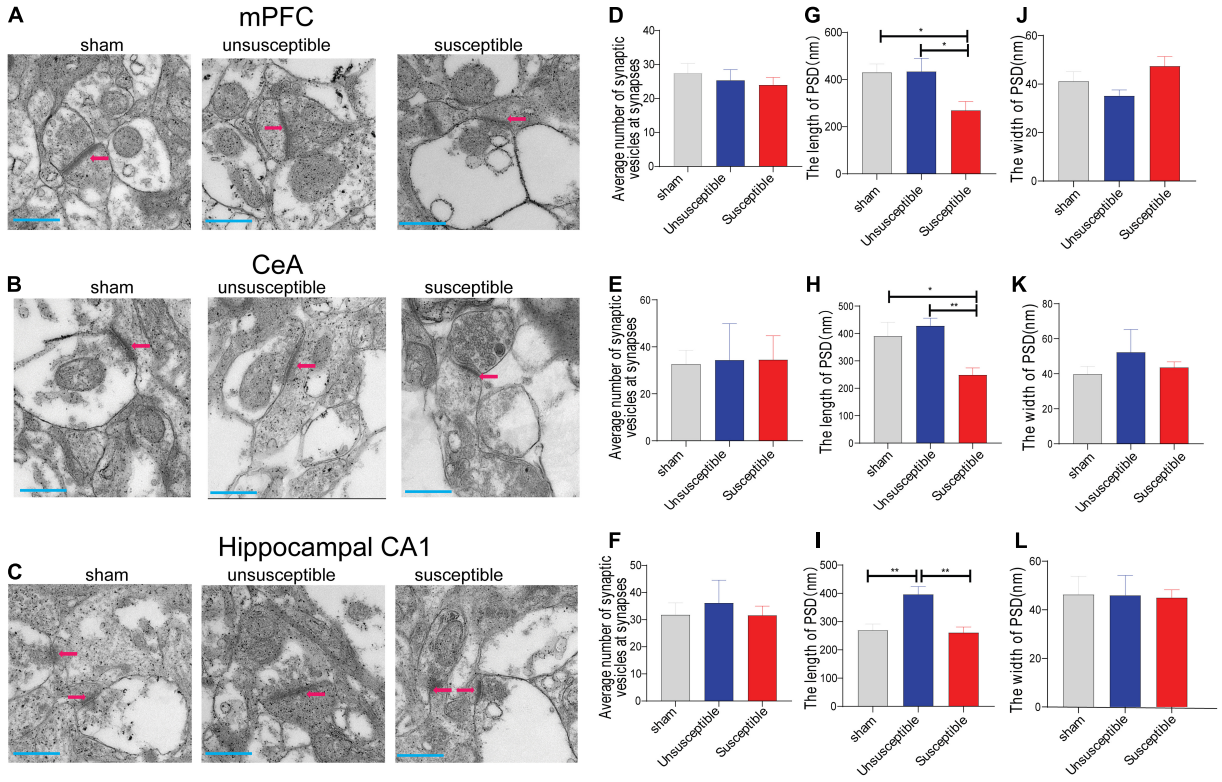

FIGURE 7
Electron microscopic images for the mPFC, CeA, and hippocampus regions after the SMIR surgery. **(A–C)** Representative synapses of the synapse ultrastructure in the mPFC, CeA, and hippocampus neurons. **(D–F)** The average number of synaptic vesicles at the synapses (n = 5 slices). one-way ANOVA, mPFC (F = 0.31, P = 0.73), CeA (F = 0.01, P = 0.99), and hippocampus (F = 0.18, P = 0.83). **(G–I)** The length of PSD (n = 5 slices). one-way ANOVA, mPFC (F = 4.54, P = 0.02), CeA (F = 6.30, P = 0.01), and hippocampus (F = 10.35, P = 0.0005). **(J–L)** The width of postsynaptic density (PSD) (n = 5 slices). one-way ANOVA, mPFC (F = 2.97, P = 0.07), CeA (F = 0.42, P = 0.66), and hippocampus (F = 0.01, P = 0.99). Scale bar = 500 nm. Results are expressed as mean ± SEM; Tukey's *post-hoc* tests; *p < 0.05, **p < 0.01.

reports (Nyffeler et al., 2007; Leuba et al., 2008; Uchimoto et al., 2014). According to previous reports, an increase in PSD-95 expression could suggest compensatory phenomena related to *de novo* PSD-95 synthesis (Nyffeler et al., 2007; Leuba et al., 2008), while an increased GluA1 level could reduce the synaptic capacity for additional GluA1 trafficking that is associated closely with cognitive function (Uchimoto et al., 2014). Moreover, these explanations were consistent with the conclusion that cognitive ability could not be enhanced simply by increasing synaptic GluA1 accumulation and, rather, the newly added receptors should possess normal properties in the dynamics (Wang et al., 2017).

The inhibitory neurotransmitter GABA is a potent regulator of pain, learning, and memory via an ongoing level of tonic inhibition mediated primarily by extra-synaptic α5GABA$_A$ receptors (Wang et al., 2012; Bravo-Hernandez et al., 2016). It has been consistently demonstrated previously that GABA concentrations are positively correlated with painful stimulus (Hasanein et al., 2008; Kupers et al., 2009). Conversely, a negative correlation exists between GABA concentrations and cognitive failure (Duncan et al., 2014; Prevot et al., 2019; Jimenez-Balado and Eich, 2021). However, the present study revealed that the level of the hippocampus α5GABA$_A$ receptor was reduced in cognitively normal pain animals, which could be explained by the fact that α5GABA$_A$ receptors also promote nociception levels by modulating the loss of GABAergic inhibition (El-Hassar et al., 2007). In addition, it was revealed that α5GABA$_A$ levels in the hippocampus were higher in the cognitively compromised pain animals compared to the cognitively normal pain animals. This was in agreement with the previous reports stating that increasing the GABA$_A$ receptor activity remarkably impeded memory (Wang et al., 2012; Whissell et al., 2013). This could be partially due to the attenuated network excitability and synaptic plasticity resulting from enhanced GABA$_A$ activity constraining the neuronal firing in the hippocampal CA1 region.

Evidence suggests that a solid operative GABAergic inhibition could dampen an increased excitation via G-protein-activated inward rectifying potassium channels (El-Hassar et al., 2007; Berry-Kravis et al., 2018). Conversely, the activation of mGluR5 receptors upon glutamate release attenuates the release of GABAergic vesicles via CB1

receptors (Ji and Neugebauer, 2011). Furthermore, multiple data demonstrate that pain and the associated cognitive deficits are complex disorders related to receptor metabolism and the functional balance between glutamate and GABA neurotransmission (Duncan et al., 2014; Zhang et al., 2019). As expected, the present study also revealed an unbalanced excitatory/inhibitory ratio in synaptic transmission. For instance, in mPFC and CeA, the alterations in the inhibitory synaptic transmission included both presynaptic and postsynaptic mechanisms, while the alterations in the excitatory synaptic transmission mainly involved the presynaptic mechanism in the rats with and without poor cognition. It should be mentioned that the changed properties of hippocampus CA1 are significantly opposite to mPFC and CeA. A possible explanation for this diversity might be due in part to the diverse effects of mGluR5 in rodent hippocampus vs. mPFC and CeA. As mGluR5 are primarily postsynaptic in hippocampus neurons to weaken synaptic functions, but are both pre- and post-synaptic in mPFC and CeA neurons known to normalize sensory gating deficit, thus improving cognitive function (Yang et al., 2021).

Spontaneous glutamatergic/GABAergic synaptic transmission is assessed based on sEPSCs/sIPSCs, while mEPSCs/mIPSCs reflect the spontaneous single synaptic response to presynaptic individual synaptic vesicles or quanta (Kaeser and Regehr, 2014; Li et al., 2016). The frequency of spontaneous synaptic transmission is often interpreted as a representation of the presynaptic release probability and the number of available synaptic vesicles, while the amplitude depends on both presynaptic released-vesicle size and the number of postsynaptic receptors (Hung et al., 2014; He et al., 2019). The present study revealed apparent discrepancies in the frequencies and amplitudes, particularly in the former, of the synaptic transmission between the CPSP rats with and without cognitive deficits. The additional data on presynaptic vesicles and PSD corroborated these discrepancies.

At the level of presynaptic status, the available data indicated that the number of synaptic vesicles could not provide adequate evidence regarding the changes in the synaptic transmission frequency linked with chronic pain and the associated cognitive deficits. As the number of synaptic vesicles in the mPFC, CeA, and hippocampal CA1 appeared to remain unaltered, the observed alterations in the frequencies of sEPSCs/sIPSCs and mEPSCs/mIPSCs were most probably due to different probabilities of glutamate or GABA quanta release from the presynaptic terminals (Hung et al., 2014; He et al., 2019). Whether the release probability of synaptic vesicles in the active zones differs distinctly among the three groups evaluated in the present study warrants further detailed investigation.

At the level of postsynaptic status, the changes in the levels of postsynaptic receptors could precisely account for the discrepancies in the amplitudes of synaptic transmission related to pain-associated cognitive deficits. Moreover, PSD in the cognitively compromised pain animals had reduced length, which confirmed that PSD was positively correlated with cognitive function (Coley and Gao, 2019). It is noteworthy that the PSD length in the hippocampal CA1 was increased in cognitively normal pain animals. Since the enhanced synaptic information communication induced by the pain stimulus would require a greater area to operate, it was supposed that the longer length of PSD was a response to the painful stimulus. Therefore, it is suggested that the release probability of presynaptic vesicles, postsynaptic receptors, and PSD could be involved in the changes and discrepancies in synaptic transmission linked to the chronic pain and the associated cognitive deficits in rats.

Five key points from the present study are noteworthy and should be addressed in future studies. First, the abundant evidence in favor of synaptic structure and function in neural circuits resulting in behavioral consequences, including pain and impaired cognition (Li et al., 2018), is not adequate to prove whether the molecular and synaptic changes in the neurons were the reasons for the pain and the associated cognitive deficits. Second, LTP, a lasting enhancement of synaptic transmission efficacy, is considered the foundation for learning and memory. Previous studies have reported comprehensive data on the synaptic plasticity mechanism of pain and cognition (Kodama et al., 2007; Xiong et al., 2020; Zhang et al., 2020; Phelps et al., 2021). Therefore, initially, the present study was also aimed to determine the spontaneous synaptic transmission and the related molecular level distinction between pain and the associated cognitive deficits. Third, a potential target for ameliorating pain and the associated cognitive deficits must be clarified further. Fourth, throughout the present study, it was apparent that no differences existed between cognitively compromised pain animals and sham controls. The literature has been consistent on a negative correlation between synaptic transmission and cognitive impairment in various models (Wong et al., 2006; Schofield et al., 2011; Ding et al., 2018; Tantra et al., 2018), while a positive correlation is reported between synaptic transmission and pain (Blom et al., 2014; Hung et al., 2014; Cordeiro Matos et al., 2015; Wang Y. J. et al., 2020). Since the cognitively compromised pain animals are with cognitive impairments, it was postulated that the alteration in the synaptic transmission related to cognitive impairments could be offset by the synaptic transmission related to chronic pain. However, this hypothesis warrants further investigation for validation. Another explanation may be that the observed changes are specific for some compensatory mechanisms involved in the cognitively normal CPSP rats. Fifth, whether female rats would exhibit the same tendency as the male rats remained to be explored.

In conclusion, the present study is a pioneer in demonstrating that both synaptic structure and function were significantly different between cognitively compromised pain rats and cognitively normal pain rats and that this

distinction was mainly due to presynaptic and postsynaptic modulation. Moreover, these findings strongly indicated that chronic pain and the associated cognitive functions could not be ameliorated simply by increasing or decreasing synaptic transmission-related receptor accumulation. Hence, although it remains unclear as to how the observed differences in these brain regions reveal any relationships between CPSP and cognitive performance, any further attempt at exploring chronic pain and the associated cognitive impairment should consider these discrepancies in the synaptic structure and function.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The animal study was reviewed and approved by the Animal Care and Use Committee, Tongji Hospital.

Author contributions

ZL and HX designed the experiments and wrote the manuscript. ZL, ZH, ZXL, TS, and WZ performed the experiments. ZL and ZH analyzed the data. All authors revised and approved the final version of the article.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The contribution of the left precuneus to emotion memory in migraine without aura patients

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Background: The impact of migraine without aura (MWoA) on cognitive function remains controversial, especially given the sparse literature on emotional memory.

Methods: Twenty seven MWoA patients and 25 healthy controls (HCs) were enrolled in this cross-sectional study. Emotional memory behavior was evaluated by combining incidental encoding with intentional encoding of five emotional categories of visual stimulus [positive valence + high arousal (PH), negative valence + high arousal (NH), positive valence + low arousal (PL), negative valence + low arousal (NL), and neutral (N)]. The recollection performance (Pr) was measured and compared. Then, the neural relevance was explored by correlating the Pr with gray matter volume (GMV) and resting-state functional connectivity (rs-FC) based on structural and functional magnetic resonance imaging.

Results: No significant differences in recollection performance or emotional enhancement of memory effect were observed. However, MWoA patients were more sensitive to the valence and arousal of emotional stimuli under incidental encoding. Significantly, the Pr-PH under incidental encoding and Pr-PL under intentional encoding were negatively correlated with the GMV of the left precuneus, and the rs-FC between the left precuneus and putamen was positively correlated with Pr-PL under intentional encoding in MWoA patients.

Conclusion: Our study demonstrated the tendency for the influence of migraine on emotional memory and revealed the left precuneus as a critical contributor to recollection performance, providing novel insights for understanding emotional memory and its neural mechanisms in MWoA patients.

KEYWORDS

migraine without aura, emotional memory, magnetic resonance imaging, gray matter volume, resting-state functional connectivity

Introduction

Migraine is a common neurological condition, with a global prevalence of approximately 15% (Headache Classification Committee of the International Headache Society, 2018). Migraine without aura (MWoA) is the most subtype classified by the International Headache Society (Silberstein et al., 2005). As a disabling headache disorder, mechanisms involved in MWoA remain unclear, and the neuropsychological impairment remains controversial (Tardiolo et al., 2019). Some studies found that several cognitive tests are unaffected by migraines (Le Pira et al., 2000; Gaist et al., 2005; Pearson et al., 2006; Camarda et al., 2007; Pellegrino Baena et al., 2018). In a population-based study of Danish twins, the cognitive performance of the twins with MWoA did not differ from non-migraineurs, and comparisons within twin pairs yielded comparable results (Gaist et al., 2005). Moreover, a retrospective single-blinded study reported that cognitive functions remained unimpaired even with a long history of MWoA (Pearson et al., 2006). However, other studies found that MWoA might lead to poor cognitive performance in executive function, processing speed, attention, and memory (Le Pira et al., 2000; Camarda et al., 2007; Pellegrino Baena et al., 2018). Such indeterminate conclusions need further exploration and research, particularly in less frequently studied high-order cognitive functions, such as emotional memory.

Previous studies have established that emotional events were better recollected than non-emotional events, a phenomenon known as the emotional enhancement of memory (EEM) effect (LaBar and Cabeza, 2006). Emotional memory paradigms were implemented to investigate the EEM effect with features such as arousal and valence (Kensinger, 2004). Arousal dichotomizes excitement and calmness, and stimuli with high arousal can enhance the initial encoding and subsequent consolidation of events by attracting attention (Mather and Sutherland, 2011). In contrast, valence refers to the positive or negative aspects of emotional stimuli that enhance memory (Dolcos and Cabeza, 2002). Memory recall from pictures or words with negative valence produces potent effects compared to positive valence, suggesting that memory may favor negative stimuli (Inaba et al., 2005; Mickley and Kensinger, 2008; Bowen and Kensinger, 2017; Bowen et al., 2018; Farris and Toglia, 2019). Studies conducted on emotional memory in Alzheimer's disease found that emotional memory was impaired, and the EEM effect was lost (Li et al., 2016). However, young adults with migraine have been reported to have a higher risk for dementia (Chuang et al., 2013). Moreover, the pathophysiology of dementia began approximately 20 years before the onset of clinical symptoms (Sperling et al., 2014). Besides, it has been shown that the prevalence of white matter hyperintensities in migraine is 38.7 ∼ 44.4% (Dobrynina et al., 2021), and the incidence of subclinical brain infarction was twice that of healthy controls (HCs) (Monteith et al., 2014). Thus, this study aims to investigate whether there is emotional memory damage in MWoA patients and further explore the underlying neural mechanism by correlating the recollection performance with voxel-wise gray matter volume (GMV) and resting-state functional connectivity (rs-FC) values, considering that the cerebral cortex and FC are crucial for brain functions (Shafiee et al., 2020; Wirsich et al., 2020).

Abbreviations: MWoA, migraine without aura; EEM, emotional enhancement of memory; NH, negative pictures with high arousal; PH, positive pictures with high arousal; NL, negative pictures with low arousal; PL, positive pictures with low arousal; N, neutral pictures; Pr, recollection performance; GMV, gray matter volume; rs-FC, resting-state functional connectivity; MoCA, Montreal Cognitive Assessment; HAMA, Hamilton Anxiety Scale; HAMD, Hamilton Depression Scale.

Materials and methods

Participants and settings

This cross-sectional study recruited 32 right-handed MWoA patients from the headache clinic of The First Affiliated Hospital of Anhui Medical University in China between June 2018 and February 2019. MWoA diagnoses were based on the headache characteristics and the International Classification of Headache Disorders 3rd edition (ICHDIII criteria) (Headache Classification Committee of the International Headache Society, 2018). Patients included in this study were18 to 60 years of age with a migraine history of 1 year before the study, experiencing a minimum of one attack per month with moderate-to-severe pain 3 months before screening. The exclusion criteria were as follows: head trauma or vascular disease; previous or current psychiatric or neurological disorders or somatoform disorders, such as depression, stroke, and dementia; substance abuse; anerythrochloropsia; magnetic resonance imaging (MRI) contraindications. Thirty-one sex-matched and age-matched volunteers from the community in the same geographical area with no personal or family history of migraine were recruited as HCs for this effort. The exclusion criteria for MWoA were also applied to HCs. Migraineurs and healthy subjects were diagnosed and screened by a specialist headache neurologist. Five MWoA patients and six HCs were excluded for analysis due to technical issues with the MRI data (motion artifact) or emotional memory data (recording issues), resulting in a final sample of 27 MWoA patients and 25 HCs.

General demographic information, including age, sex, and education level, was collected from all participants. Montreal Cognitive Assessment (MoCA), Hamilton Anxiety Scale (HAMA), and Hamilton Depression Scale (HAMD) were used to evaluate global cognitive function, anxiety, and depression, respectively. The emotional memory test and MRI scans were performed during the inter-migraine period, a 2-day interval during the absence of acute migraine attacks. The characteristics of migraine, such as duration of

disease, monthly frequency and duration of attacks, and pain intensity/impact, were collected.

Emotional memory behavioral test

Stimulus

Two hundred pictures were selected from the International Affective Picture System (Lang et al., 2010), a widely used collection of color photographs featuring the two independent emotional properties of arousal and valence. Both properties ranged from 1 to 9, where 1 corresponded to a very negative valence or low arousal state, and 9 corresponded to a very positive or high arousal state. The valence was considered negative, positive, and neutral for ratings of ≤ 4, ≥ 6.0, and 4.5–5.5, respectively. Similarly, the arousal was considered high, low, and neutral for ratings of ≥ 6.0, ≤ 4.0, and 4–6, respectively. According to valence and arousal ratings, the pictures were divided into five categories: positive with high arousal (PH), positive with low arousal (PL), neutral (N), negative with high arousal (NH), and negative with low arousal (NL) (Li et al., 2020). The neutral category excluded high/low arousing and valence images. These pictures were depicted in 2 lists of 100 stimuli. Then each list was divided into two 50-picture subgroups. Each subgroup consisted of 10 PH, 10 PL, 10 N, 10 PL, and 10 PL. For each list, one subgroup (50 pictures) was presented during the encoding phase, and both subgroups (100 pictures—50 seen and 50 unseen) were presented during the retrieval phase (**Figure 1**). These pictures included humans and landscapes and were presented in counterbalance.

Apparatus

The E-prime v.2.0 software (PST Inc., Sharpsburg, PA, USA) was used to present the stimuli and record the participants' responses on a laptop computer.

Procedure

The procedure began when the participants were ready and acclimatized to the new environment, a room for neuropsychological testing. The behavioral test was divided into incidental and intentional sessions according to encoding. Each session had an encoding phase, where participants were asked to immediately identify whether the main object shown in a picture was a person (categorization task). During this phase, the stimulus was presented for 2,000 ms, and the stimulus interval was 500 ms. Then, there was a 30 min delay between the encoding and retrieval phases. During the retrieval phase, the remember/know procedure was employed to estimate recollection and familiarity directly (Yonelinas, 1994). Participants were asked to identify whether the image presented was old (seen during the encoding phase) or new (or not). If the pictures were considered old, they were then asked whether they recollected the details of the images (recollection) or were only familiar with the pictures (familiarity) based on their memory differences (recognition task) (Quamme et al., 2010). Some quality control methods were performed to ensure that each subject could distinguish recollection from familiarity as accurately as possible and follow the same criteria. Practice examples were provided with on-screen instructions before the test to ensure that the participants understood each task and the difference between recollection and familiarity. Moreover, participants were asked to describe the criteria they used in the retrieval task at the end of the test. During the incidental session, participants were unaware of the later retrieval task in the encoding phase. However, participants were asked to memorize the pictures carefully when encoded during the intentional session. The intentional session began after the completion of the incidental session (**Figure 1**). Investigators and participants were double-blinded for the test.

Magnetic resonance imaging acquisition

All MRI data were acquired on a General Electric 750 w 3.0 T MRI scanner (General Electric, Waukesha, WI, USA) with a 24-channel head coil. The MRI protocol included the acquisition of three-dimensional T1-weighted (3D T1) high-resolution structural images, resting-state blood oxygen level-dependent scans, and axial T2-weighted and FLAIR images. The included participants did not show structural abnormalities on the MRI examination. The BRAVO (brain volume) sequence [repetition time (TR) = 8.5 ms, inversion time (TI) = 450 ms, echo time (TE) = 3.2 ms, 188 slices, no slice gap, slice thickness = 1 mm, field of view (FOV) = 256 × 256 mm^2, matrix size = 256 × 256, and flip angle = 12°] was used to acquire the 3D T1 images. An echo-planar imaging sequence (TR = 2,000 ms, TE = 30 ms, slice gap = 1 mm, slice thickness = 3 mm, FOV = 220 × 220 mm^2, matrix size = 64 × 64, and flip angle = 90°) was used to acquire resting-state functional MRI (rs-fMRI) scans.

Structural magnetic resonance imaging pre-processing

Structural 3D T1 images were pre-processed using the VBM8 (voxel-based morphometry)[1] toolbox in SPM8 (Statistical Parametric Mapping)[2] in Matlab (Mathworks, Natick, MA). First, we visually inspected all structural images to screen for anatomical abnormalities or artifacts. The standard brain templates were used to segment the image into gray matter (GM), white matter (WM), and cerebrospinal fluid (CSF) volumes. A diffeomorphic anatomical registration algorithm (DARTEL) toolbox was used to co-register structural MR images. For each subject, a flow field was created for

1 https://neuro-jena.github.io/software.html#vbm

2 http://www.fil.ion.ucl.ac.uk/spm/

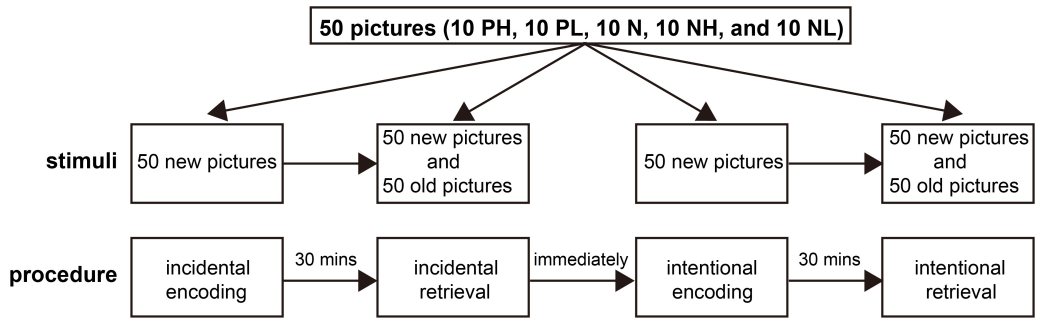

FIGURE 1
The procedure of the emotional memory paradigm.

wrapping scans onto the template. Then, gray matter images were normalized spatially to the Montreal neurological institute (MNI) coordinates using these data. Subsequently, the images were resampled at 1.5 × 1.5 × 1.5 mm voxel size and smoothed with an 8 mm full-width half-maximum (FWHM) Gaussian kernel to construct a DARTEL template (Cheng et al., 2015).

Resting-state functional magnetic resonance imaging data pre-processing

The rs-fMRI data were pre-processed with DPABI (Data Processing and Analysis of Brain Imaging)[3] (Yan et al., 2016), a MatLab toolbox. First, we accounted for the magnetic field instability, and the initial 10 volumes were removed for each scan. Then, images within each scan were realigned using 1.5 mm and 1.5° movement thresholds to correct motion between time points. The frame-wise displacement was calculated, and other covariates, such as estimated motion parameters and the WM and CSF signals, were regressed. The data set was then bandpass filtered between 0.01 and 0.08 Hz. Finally, the individual structural images were co-registered to the mean functional image. Then, the structural and functional images were normalized to the MNI space using the DARTEL toolbox. These images were resliced to a 3 × 3 × 3 mm voxel and spatially smoothed with a 6 mm FWHM Gaussian kernel. The DPABI software was used to define a seed region using automatic anatomical labeling (AAL) (Tzourio-Mazoyer et al., 2002), and the functional connectivity (FC) was calculated between the seed region and the rest of the brain. The correlation coefficients (r) were transformed into Fisher z-scores to obtain normally distributed values.

Statistical analysis

Demographic data analysis

This was the primary analysis of the data. Statistical analysis was performed using SPSS 23.0 software package (SPSS, Chicago, III). Normally and skewed distributed variables were reported as mean ± standard deviation and median (25th, 75th percentiles), respectively. We assessed normality and compared general demographic characteristics between the two groups using a χ^2-test for sex and Mann-Whitney *U*-tests for age, education level, MoCA, HAMA, and HAMD scores. Statistical significance was set at a two-tailed p-value < 0.05.

Emotional memory behavioral test

The hit (Hit) and false alarm (FA) rate was calculated as the ratio of the sum of remember and know judgments given to old and new pictures, respectively. This study used the difference between Hit and FA judged by memory as the recall score index, expressed as Pr (Koen and Yonelinas, 2016). *A priori* power analysis was performed using G*Power 3 program to compute the necessary sample size. Alpha, effect size, and power (1 – β) were set at 0.05, 0.25, and 80%, respectively (Cona et al., 2015). The sample size of 25 individuals in each group was considered sufficient. A 5 × 2 mixed-factorial covariance analysis was performed on Pr with the stimuli emotional categories (5 categories, PH, PL, N, NH, and NL) as the within-subject factors and the participant groups (2 groups) as the between-subject factors with the MoCA, HAMA, and HAMD scores as covariates. The Bonferroni correction was used for *post hoc* multiple comparisons. Investigators responsible for data analyses were blinded to patient grouping.

Gray matter volume analysis

Voxel-wise analysis of within-group correlation in GMV and Pr was conducted with a multiple regression model. Sex, age, education level, total intracranial volume, MoCA, HAMA, and HAMD scores were used as covariates.

Functional connectivity analysis

The significant brain region identified using correlation analyses between GMV and Pr in MWoA patients was used as the seed region in the rs-FC analysis. Voxel-wise analysis was performed on the within-group correlation with the rs-FC values and Pr using a multiple regression model. Sex, age,

3 https://rfmri.org/dpabi

education level, head motion, MoCA, HAMA, and HAMD scores were set as covariates.

In GMV and FC analysis, we set the statistical threshold at p-value < 0.05 (cluster level, FWE corrected) with an extent threshold of 50 voxels. Significant clusters were automatically identified using the Xjview toolbox.[4] Each significant cluster in GMV or rs-FC correlation analysis was extracted for each subject and used for the region of interest analysis.

Results

General demographic results

Table 1 contains demographic and clinical information of the study participants. There were no significant differences in gender, age, and education degree between the two groups ($p > 0.05$). In MWoA patients, the MoCA score was lower, and the HAMA and HAMD scores were higher than HCs ($p < 0.05$).

TABLE 1 General demographic information of participants.

	MWoA ($n = 27$)	HC ($n = 25$)	p
Demographic characteristics			
Age (years)	28 (25, 36)	28 (23, 38.5)	0.324
Sex (male/female)	6/21	10/15	0.165
Education degree (years)	16 (9, 17)	16 (12, 17)	0.899
Cognitive			
MoCA	28 (25, 28)	28 (27, 29)	*
Emotion			
HAMA	5 (1, 10)	1 (0.5, 2)	*
HAMD	6 (1, 10)	1 (0, 2)	*
Migraine characteristics			
Duration of migraines in years	10 (6, 14)	——	——
Monthly frequency of migraine attacks	4 (3, 4)	——	——
Attack duration	12 (12, 12)	——	——
NRS	5 (6, 7)	——	——
HIT-6	62 ± 7.1	——	——

*$p < 0.001$. The monthly frequency of migraine attacks was the mean frequency of 3 months before the participation interview. Pian intensity was calculated as the mean numeric rating scale (NRS: 0 = no pain to 10 = unbearable pain) score for the days with a headache. HIT-6, headache impact test, ranges from 36 to 78; a higher score represents a more severe headache.

Emotional memory behavioral test results

Incidental encoding

For incidental encoding, the group x emotion interaction was significant [$F(4, 188) = 2.449$, $p = 0.048$] (Figure 2A). The result suggested a bias in collection performance between the two groups. Then, further analysis showed that the simple main effect of emotion (within-subject factors), but not group (between-subject factors), was significant. The PH, NH, and NL stimuli were better discriminated compared to neutral stimuli in MWoA patients ($p = 0.01$, $p < 0.001$, and $p = 0.001$) and HCs ($p = 0.023$, $p < 0.001$, and $p = 0.006$). Therefore, these results illustrated the EEM effect in MWoA patients and HCs. Moreover, MWoA patients ($p = 0.009$) and HCs ($p = 0.001$) recollected the PH stimuli better than the PL stimuli; however, only the MWoA patients ($p < 0.001$), but not HCs ($p > 0.05$), recollected the NH stimuli better than the NL stimuli. Similarly, the patients ($p < 0.001$) and HCs ($p < 0.001$) recollected the NL stimuli better than the PL stimuli; however, only the patients ($p < 0.001$), but not HCs ($p > 0.05$), recollected the NH stimuli better than PH stimuli. Besides, NH stimuli were better recollected than PL stimuli in both groups ($p < 0.001$) (Figures 3A,B). In brief, the results showed that MWoA patients were more sensitive to emotional stimuli composition (valence and arousal) (Table 2). Besides, no significant main effect of group [$F(1, 47) = 0.663$, $p = 0.419$] or emotion [$F(4, 188) = 0.933$, $p = 0.446$] was observed.

Intentional encoding

For intentional encoding, the main effect of group [$F(1, 47) = 0.814$, $p = 0.372$] or emotion [$F(4, 188) = 0.144$, $p = 0.965$], and the group x emotional interaction [$F(4, 188) = 0.125$, $p = 0.973$] was insignificant (Figures 2B, 3C,D and Table 2).

Correlation between gray matter volume and recollection performance

In MWoA patients, the GMV of the left precuneus [cluster size = 601 voxels, peak MNI coordinate x/y/z = −6/−78/57, peak $T = 5.72$, and partial correlation coefficient (r) = −0.79, $p < 0.001$, Figure 4] was negatively correlated ($p < 0.05$, FWE corrected) with the Pr-PH under incidental encoding. Interestingly, the GMV of another cluster in the left precuneus (cluster size = 1144 voxels, peak MNI coordinate x/y/z = −7.5/−61.5/39, peak $T = 5.75$, and $r = -0.776$, $p < 0.001$, Figure 5) were negatively correlated ($p < 0.05$, FWE corrected) with Pr-PL under intentional encoding. No other significant correlations between the GMV and Pr were observed in MWoA patients. However, no significant correlations between the GMV in the left precuneus and Pr were found for HCs in voxel-wise analysis. Moreover, the GMV of the significant brain region derived using correlation analysis was extracted in MWoA patients, and partial correlation analysis

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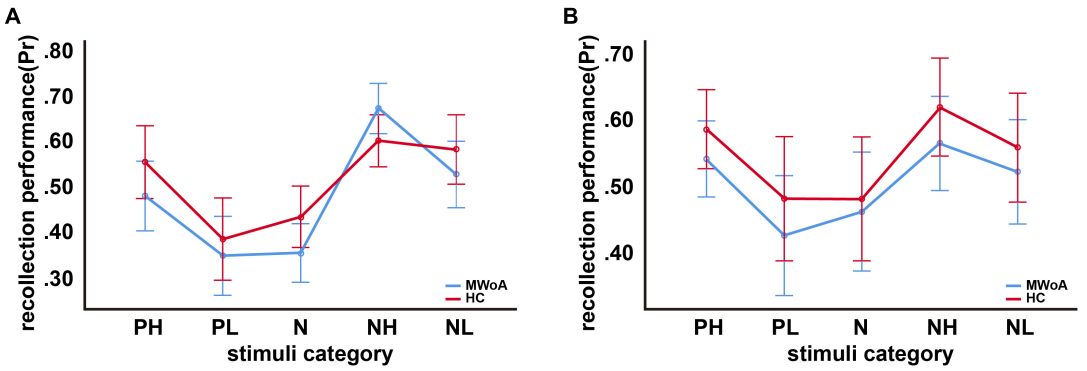

FIGURE 2
Recollection performance as a function of picture type. **(A)** Incidental encoding, **(B)** intentional encoding. Note that Pr represents recollection performance, which varies from 0 (no discrimination between old and new pictures) to 1 (perfect discrimination). Bars represent estimated marginal means ± standard deviations.

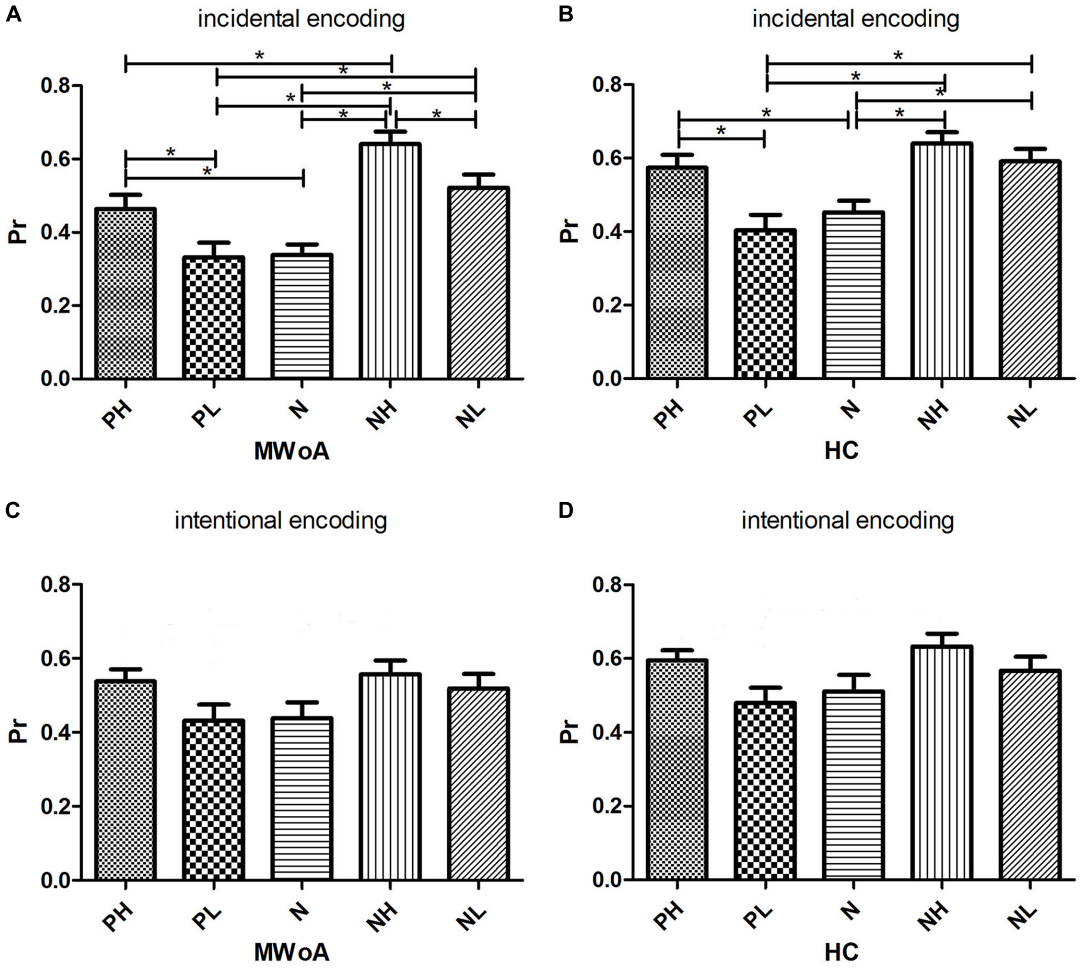

FIGURE 3
Histograms of simple main effects of emotion on Pr value within the group. **(A,B)** The comparison of Pr between the 5 categories of stimuli under incidental encoding in MWoA patients and HCs, respectively. **(C,D)** The comparison of Pr between the 5 categories of stimuli under intentional encoding in MWoA patients and HCs, respectively. *$p < 0.05$. Bars represent means ± standard errors.

TABLE 2 The Pr value of patients with MWoA and healthy controls.

	Incidental encoding		Intentional encoding	
	MWoA	HC	MWoA	HC
N	0.338 ± 0.151	0.452 ± 0.161	0.438 ± 0.224	0.511 ± 0.222
PH	0.464 ± 0.200	0.574 ± 0.174	0.538 ± 0.167	0.595 ± 0.137
PL	0.332 ± 0.209	0.403 ± 0.210	0.432 ± 0.224	0.480 ± 0.206
NH	0.641 ± 0.176	0.640 ± 0.150	0.557 ± 0.194	0.633 ± 0.173
NL	0.522 ± 0.187	0.591 ± 0.168	0.519 ± 0.204	0.567 ± 0.191

was performed with the Pr in HCs. However, significant results were not identified ($p > 0.05$).

Correlation between resting state functional connectivity and recollection performance

The AAL atlas defined the left precuneus as the seed region for rs-FC analysis. In MWoA patients, the rs-FC between the left precuneus and the left putamen (cluster size = 62 voxels, peak MNI coordinate x/y/z = 33/−12/−6, peak $T = 5.73$, and $r = 0.817$, $p < 0.001$, **Figure 6**) was positively correlated ($p < 0.05$, FWE corrected) with the Pr-PL under intentional encoding. In HCs, the rs-FCs between the left precuneus and several brain regions (the lingual gyrus, calcarine sulcus, superior temporal gyrus, paracentral lobule, and postcentral lobule) were positively correlated ($p < 0.05$, FWE corrected) with the Pr-PH under incidental encoding (**Table 3**). The rs-FC between the left precuneus and inferior parietal cortex was also positively correlated ($p < 0.05$, FWE corrected) to the Pr-PH under intentional encoding (**Table 3**).

Discussion

Recollection and familiarity are two separate processes underlying emotional memory. Based on the dual-process approach, familiarity reflects a classical signal-detection process, and recollection reflects a threshold process (Yonelinas, 2001). The recollection, but not familiarity, decreases over time, particularly over short intervals, and the decline of the recollection component causes memory loss (Kishiyama et al., 2005; Yang et al., 2016). Therefore, the analysis focused on the recollection component of emotional memory. In this study, the MWoA patients and HCs exhibited an EEM effect under incidental encoding. Recollection benefited from extreme valence and arousal and increased the distinction during the encoding step (Alonso et al., 2015). Kensinger et al. (2007) demonstrated a similar enhancement for negative and high arousal stimuli compared to neutral ones. Under intentional encoding, both groups' EEM effect was missing, consistent with previous reports in young adults (Kensinger et al., 2005, 2007). For intentional encoding, participants attempted to focus their cognitive resources on all memorization items, including neutral stimulus, by prompting subsequent retrieval tasks. For incidental encoding, participants were uninformed of subsequent retrieval tasks; therefore, they could not allocate similar resources, resulting in a subjective connection toward different stimuli/items. MWoA patients might be benefiting from the intentional encoding instructions and elaborate encoding strategies, similarly to the HCs (Kensinger et al., 2005). Understanding the enhancement of emotional characteristics on memory in MWoA patients could help develop interventions to prevent dementia and promote healthy aging (Alonso et al., 2015). Notably, MWoA patients exhibited better recollection performance for PH than NH and NH than NL under incidental encoding. These differences were not observed in HCs, indicating that patients with MWoA were more susceptible to valence and arousal of emotional stimuli. However, we did not find a deterioration in recollection performance or EEM effect. The reasons for this are likely twofold. On the one hand, the limbic circuitry, comprised of the amygdala and hippocampus, is not only responsible for emotions, learning, and memory but is also implicated in experiential aspects of pain. It has been demonstrated that the chronification of pain was activity-induced plasticity of limbic cortical circuits leading to neocortex reorganization, during which the representation of pain gradually shifts from sensory to the memory of pain and/or the inability to extinguish painful memories (McCarberg and Peppin, 2019). One the other hand, previous studies reported that MWoA patients showed enhanced brain activation toward emotional stimulation (Wilcox et al., 2016) and were more sensitive to negative stimuli (Wang et al., 2017). In particular, the unpleasant category of IAPS images included highly arousing mutilation and attack images (Lang et al., 2010). Although poor global cognitive functions were observed in migraine patients, they may not capture changes in general intellectual functions or might be sensitive to changes in specific cognitive domains (Gates et al., 2019). Thus, a tendency for influence of MWoA on emotional memory was considered.

In MWoA patients, we observed correlations between the GMV of the left precuneus and recollection performance. However, the precuneus is a core region of the default mode network (DMN) involved in episodic memory (Buckner et al., 2008). The abnormalities in the precuneus might affect information transfer, multimodal integration, and pain sensitivity and processing in MWoA patients (Zhang et al., 2016). The left precuneus might be damaged, remodeled, and involved in a compensation mechanism during pain management, which might explain the negative correlation between the GMV of the left precuneus and

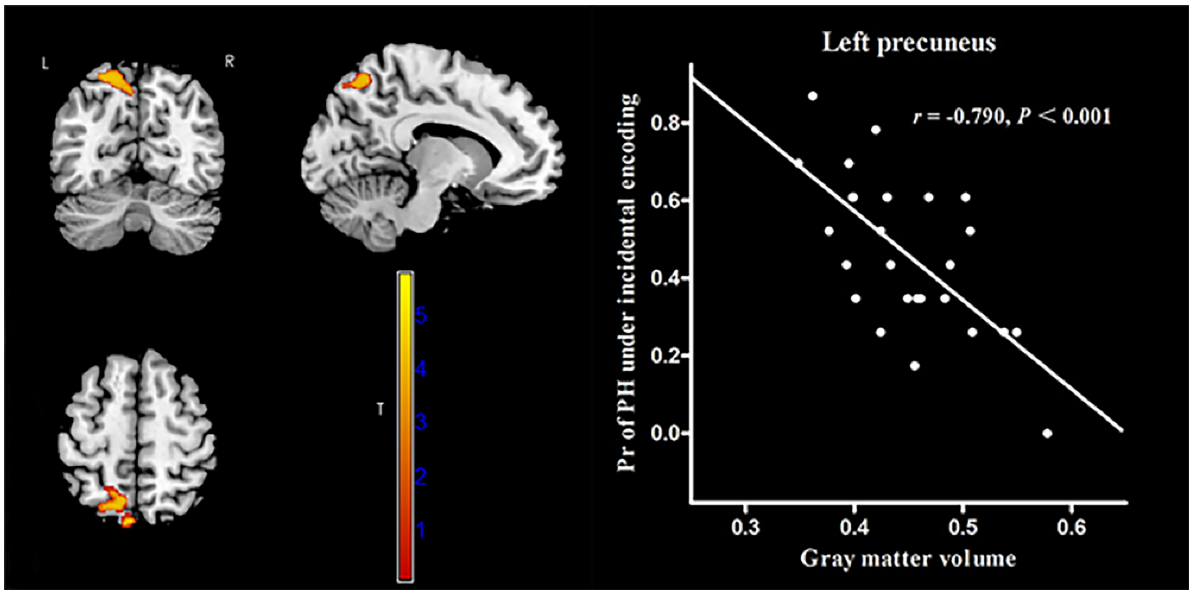

FIGURE 4
Correlation between voxel-wise gray matter volume in the left precuneus and the Pr-PH (positive stimulus with high arousal) under incidental encoding in MWoA patients. $p < 0.05$, cluster-level FWE corrected. Scatter plot of ROI-based partial correlation analysis between gray matter volume in the left precuneus and the Pr-PH.

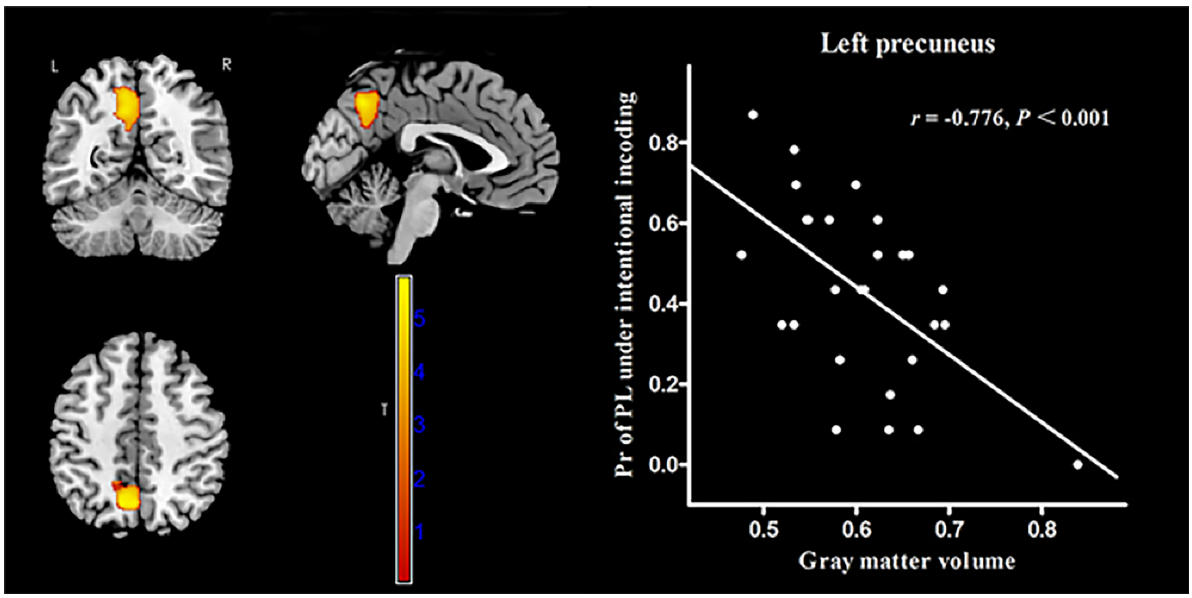

FIGURE 5
Correlation between voxel-wise gray matter volume in the left precuneus and the Pr-PL (positive stimulus with low arousal) under intentional encoding in MWoA patients. $p < 0.05$, cluster-level FWE corrected. Scatter plot of ROI-based partial correlation analysis between gray matter volume in the left precuneus and the Pr-PL.

recollection performance. Moreover, the rs-FC between the left precuneus and the left putamen positively correlated with the recollection performance. The putamen is involved in cognitive, emotional, and reward processing (Haber and Knutson, 2010; Ghandili and Munakomi, 2022) and connects to the components of the DMN (Byrne et al., 2019). In this study, the FC of this intra-reward system was correlated with the recollection performance in MWoA patients.

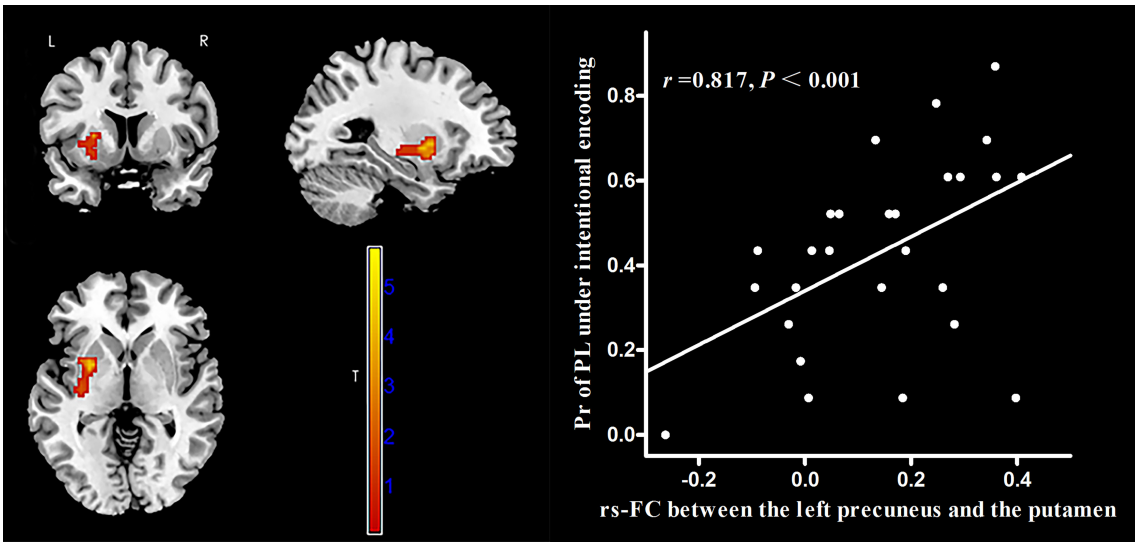

FIGURE 6
Correlation between voxel-wise rs-FC of the left precuneus and the left putamen and the Pr-PL (positive stimulus with low arousal) under intentional encoding in MWoA patients. $p < 0.05$, cluster-level FWE corrected. Scatter plot of ROI-based partial correlation analysis between rs-FC and the Pr-PL.

TABLE 3 Correlation between the recollection performance and the rs-FC of the left precuneus and brain regions in healthy controls.

Brian regions	Peak *T*	cluster size	Peak MNI (mm)		
			x	y	z
Incidental encoding pR-PH					
Lingual gyrus	4.84	115	−15	−87	−18
Calcarine	5.17	116	18	−96	9
Superior temporal	6.03	93	−51	−21	6
Paracentral lobule	5.57	196	−6	−42	53
Postcentral	5.42	61	24	−30	57
Intentional encoding pR-PH					
Inferior parietal	5.93	93	−42	−60	51

$p < 0.05$, cluster level FWE corrected. Coordinates of peak voxels (x, y, z) are given in MNI space.

GMV in the left precuneus and the rs-FC between the left precuneus and the left putamen were not correlated to the recollection performance in HCs. The precuneus, putamen, and reward systems participate in pain processing during migraine (Tanasescu et al., 2016; Zhang et al., 2016; Porreca and Navratilova, 2017). Therefore, we believed these associations might result from the pathological mechanism of the disease itself. In HCs, the rs-FCs between the left precuneus and several brain regions (the lingual gyrus, calcarine sulcus, superior temporal gyrus, paracentral lobule, and postcentral lobule) were positively correlated with recollection performance. The lingual gyrus and calcarine sulcus belong to the visual cortex, (Huff et al., 2022). Gao et al. (2019) revealing that the superior temporal gyrus was a brain region responsible for audiovisual affective and emotional processing. Moreover, the inferior parietal lobule is a component of the ventral attention network (Corbetta et al., 2008). It indicated that information from various brain areas/networks was integrated to successfully perform the task in HCs. When patients are exposed to a migraine attack, changes in neurobiological progression might lead to changes in mood, vision, attention, cognition, and behavioral regulation. The integration of the necessary information for behavior might be disturbed. However, neural reserve and compensation support the cognitive reserve (Anthony and Lin, 2018). In MWoA patients, the performance of emotional memory might be maintained because of compensatory contribution from the left precuneus.

This study had several limitations. First, it was a cross-sectional study with a sex-skewed sample and a widely spread age group. The included migraine patients were only without

aura, and their education level was relatively high. Longitudinal studies with expanding sample diversity, such as migraine subtypes and left-hand participants, are needed in the future to observe the applicability of the current results. Second, although we controlled for several potential confounders, residual confounding factors could exist. Third, the behavioral test for emotional memory was within a laboratory setting, and tests are required in real-life environments in the future. Moreover, the recollection performance may differ when using different encoding-retrieval interval times (Yang et al., 2016). Finally, for the rs-FC analyses, the left precuneus was focused on and set as the seed region. Other brain regions should also be incorporated in future studies.

Conclusion

In conclusion, our results revealed the tendency for the influence of migraine on emotional memory. Although these impacts were insufficient to indicate deterioration in recollection performance and EEM effect, the patients with MWoA were more sensitive to valence and arousal of emotional stimuli under incidental encoding. Moreover, a difference was found in the structural and functional contributions of the left precuneus in recollection performance between MWoA patients and HCs, indicating its crucial role in emotional memory. These findings help recognize the association between migraine and emotional memory and its neural correlations. It might provide novel insights into early interventions in preventing cognitive decline because of migraine.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Institutional Review Board and Ethics Committee of the First Affiliated Hospital of Anhui Medical University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YY conceived and designed the study. ML and XL drafted and revised the manuscript. WZ and ZG participated in data collection, cognitive function assessment, and visualization. JZ and HW helped with data analysis. XW, SZ, and KW participated in case collection and scale assessment. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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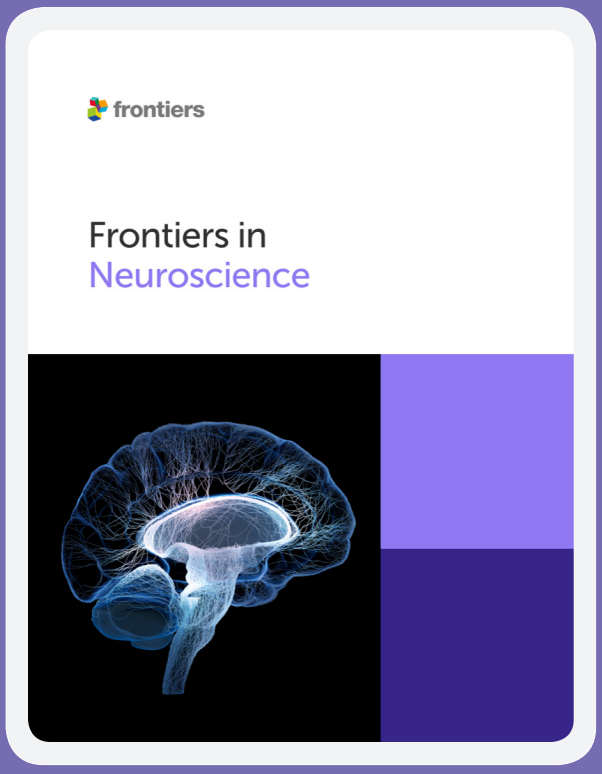

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