INTERPLAY OF STRESS, PAIN AND PSYCHIATRIC DISEASES

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INTERPLAY OF STRESS, PAIN AND PSYCHIATRIC DISEASES

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Bi-Directional Relationships Between Psychological Symptoms and Environmental Factors in Early Adolescence

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Aim: Bi-directional relationships between various environmental factors and psychological symptoms can be seen from childhood to adolescence; however, there has been little prospective cohort study, which investigated the relationships simultaneously. In this study, we first distinguished specific psychological symptoms from general psychopathology using bifactor modeling and then tested the relationships between psychological symptoms and environmental factors from childhood to early adolescence using a structural equation model (SEM).

Methods: The analyses were based on Tokyo TEEN Cohort (TTC) data collected between October 2012 and March 2016. We obtained self-reported psychological symptoms and environmental factors from both parents and children (at their ages of 10 and 12). Participants were 3,171 children aged 10 [girls = 1,487 (46.9%), mean age, SD = 10.2, 0.28] and subsequently 12 (N = 3,007, follow-up rate 94.8%, mean age, SD = 12.2, 0.31) from three municipalities in Tokyo area.

Results: The best-fit symptom models included four unique factors and general psychopathology as the common factor. Combining the good fit bifactor model and the SEM, positive relationships between symptoms and environmental factors at the same waves and some bi-directional relationships were found. Especially, general psychopathology at age 10 was associated with bullying at age 12 and parental depressive symptoms at age 10 with general psychopathology at age 12. However, some negative relationships such as bullying/bullied involvement and later psychological symptoms were also seen.

Conclusion: By using the newly introduced methodology, our results were partly consistent with previous literature. Further studies are needed to validate this

methodology and accelerate the findings regarding the emergence of psychological symptoms and the impact of environmental factors from childhood to early adolescence.

Keywords: child, young adolescent, cohort, longitudinal analysis, bifactor analysis, structural equation modeling

INTRODUCTION

Environmental influences are known to be the key factors for the emergence of psychological symptoms and psychiatric disorders (1, 2). Clinical and population-based investigations have shown that stressful experiences from childhood to adolescence are common risk factors for the onset of psychiatric disorders in their later lives (3, 4). For example, bully victimization increases the odds of the emergence of depressive episodes (5–7), anxiety (8), and psychotic disorders (9) in later life. These relationships were also observed for psychological symptoms in the general population (10–12).

Parental depressive symptoms (13, 14) and parenting style (15) are also key factors for children's mental health. A cohort study from U.S. NESARC (n = 43,093) showed that a history of physical, emotional, or sexual abuse in the first 17 years of life was associated with both internalizing and externalizing psychopathology in adults (16). However, there are limited researches exploring the relationships between multiple environmental factors and psychological symptoms simultaneously in a prospective cohort study. Testing such relationships simultaneously could provide additional information about whether a symptom was indeed driven by a specific environmental factor and vice versa. This will also provide a better understanding of the emergence and progression of psychopathology during adolescence (16, 17).

Since the presence of one psychological symptom often comes with others that belong to another clinical category, the potential comorbidity of psychiatric disorders and psychological symptoms should be considered in testing multiple psychological symptoms (18, 19). Bifactor modeling has become a popular method to differentiate specific symptoms from common general psychopathology based on the presence of other psychological symptoms (20–24). A UK ROOTs study (n = 1,159) applied this model for self-reported depression and anxiety at age 14 years and distinguished the specific factors of hopelessness, restlessness, and general worrying by introducing a general factor (23). Another study using a functional magnetic resonance imaging technique showed that there were strong associations between the latent variables of anxiety and amygdala-related connectivity, and between that of irritability and insular-related connectivity (24). In addition, the general psychopathology, namely, the p factor, which is extracted from a bifactor model with various symptoms, was reported and well-replicated (20, 22). However, no prospective cohort study has investigated the relationships between environmental factors and general psychopathology factors and other psychological factors.

In this study, we intended to investigate the relationships between multiple environmental factors and psychological symptoms simultaneously in Tokyo teen cohort (TTC), a population-based prospective cohort in Tokyo, Japan (25, 26). In this cohort, we measured a variety of psychological symptoms such as depressive symptoms and psychotic experiences from children at their age of 10 and 12 and internalized and externalized behavioral problems such as anxiety, somatoform, and aggressive behaviors from their main caregivers. Various environmental factors such as parental depressive symptoms, parenting style, and bullying/bullied involvement at the two waves were also measured. These features of the TTC database enabled us to test the associations between environmental factors and psychological symptoms evaluated by bifactor modeling using structural equation modeling (SEM). We hypothesized that after controlling the correlations between psychological symptoms and environmental factors, general psychopathology at age 10 would have a relationship with some environmental factors at age 12 and vice versa.

METHODS

Participants

The sample of TTC was recruited from three municipalities (Setagaya-ku, Mitaka-shi, and Chofu-shi) in the metropolitan area of Tokyo (25, 26). The data were obtained between 2012 and 2015 for wave 1 (N = 3,171, girls = 1,487, age: mean [SD] = 122.1 [3.3] months; **Table 1**) and between 2014 and Mach 2017 for wave 2 (N = 3,007, girls = 1,418, age: 146.0 [3.7] months; follow-up rate 94.8%).

There were no statistical differences in demographic characteristics including age, sex, or paternal and maternal education between those who took part in the followed-up study and those who did not ($p > 0.05$). Ethical approval for this study was obtained from the Ethical Committee of Tokyo Metropolitan Institute of Medical Science (number: 12-35), the University of Tokyo (number: 10057), and SOKENDAI (the Graduate University for Advanced Studies, number: 2012002). The children's main caregivers (usually mothers) gave written informed consent. The data analysis was conducted between September 2018 and November 2019.

Surveys

We used TTC child and parent self-report questionnaires for environmental factors and psychological symptoms at both wave 1 and 2. 98.3 and 97.9% of the respondents (main caregivers) were the participants' mothers in wave 1 and 2, respectively. All the examiners had ensured anonymity to all participants before they gave any responses. In order to maintain confidentiality and reliability of the sensitive questions, the printed questionnaires [the child behavior checklist (CBCL), the general health questionnaire (GHQ-28), socio-economic status, the short mood and feeling questionnaire (SMFQ), the adolescent psychotic-like symptom screener (APSS), and bullying involvement] were all put in concealed envelopes. In addition,

TABLE 1 | Descriptive statistics of TTC at ages 10 and 12.

		Age 10 (n = 3,171)		Age 12 (n = 3007)		Cohen's D[a]	p[a]
		Number/*mean*	%/*SD*	Number/*mean*	%/*SD*		
Age (month)		*122.1*	*3.3*	*146.0*	*3.7*		
Sex (female)		1,487	46.9%	1,418	47.2%		
Psychological symptoms							
CBCL	Internalized score	*53.46*	*8.72*	*52.16*	*8.8*	0.18	<0.01
	Externalized score	*51.41*	*8.50*	*49.65*	*8.24*	0.28	<0.01
SDQ	Emotional symptom	*1.21*	*0.55*	*1.17*	*0.50*	0.06	<0.01
	Conduct problems	*1.21*	*0.54*	*1.19*	*0.52*	0.04	0.03
	Hyperactivity/inattention	*1.23*	*0.59*	*1.17*	*0.51*	0.11	<0.01
	Peer relationship	*1.17*	*0.51*	*1.18*	*0.52*	0.02	0.28
	Prosocial behavior	*1.43*	*0.71*	*1.5*	*0.76*	0.10	<0.01
SMFQ		*4.76*	*4.58*	*3.84*	*4.49*	0.17	<0.01
APSS		*0.87*	*0.91*	*0.83*	*0.99*	0.03	0.12
Bully involvement							
Bullied	Left out	492	16.1%	213	8.6%	0.17	<0.01
	Called mean names	595	19.5%	260	10.5%	0.19	<0.01
	Hit lightly	351	11.5%	130	5.2%	0.15	<0.01
	Hit strongly	194	6.3%	42	1.7%	0.18	<0.01
	Things taken	76	2.5%	35	1.4%	0.06	<0.01
	Not bullied	2081	68.0%	2022	81.3%	0.23	<0.01
Bullying	Left out	21	7.0%	113	4.5%	0.06	<0.01
	Called mean names	184	6.1%	98	3.9%	0.08	<0.01
	Hit lightly	133	4.4%	60	2.4%	0.07	<0.01
	Hit strongly	42	1.4%	10	0.4%	0.08	<0.01
	Things taken	15	0.5%	7	0.3%	0.02	0.44
	Not bully	2584	86.1%	2273	90.9%	0.10	<0.01
Parental depressive symptoms							
K6		*2.94*	*3.33*				
GHQ-28	Depression-related 7 items				*8.04*	*4.28*	
Educational level of father							
	High school or less	542	18.0%				
	2-year college	409	13.6%				
	4-year university	1692	56.1%				
	Graduate university	374	12.4%				
Educational level of mother							
	High school or less	524	16.7%				
	2-year college	1391	44.2%				
	4-year university	1126	35.8%				
	Graduate university	105	3.3%				
Annual household income (10 000 yen)							
	0–299	142	4.7%				
	300–599	763	25.0%				
	600–999	1224	40.2%				
	1000+	917	30.1%				

CBCL, the child behavior checklist; SDQ, the strength and difficulties questionnaire; SMFQ, the short mood and feeling questionnaire; APSS, the adolescent psychotic-like symptom screener; K6, the Kessler psychological distress scale; GHQ-28, the 28-item version of the general health questionnaire.
[a]*Paired t-test was performed between age 10 and 12.*

the participants were also informed that a third person would be assigned to code their responses.

Psychological Symptom Measurement

For psychological symptoms, CBCL (27) and the strength and difficulties questionnaire (SDQ) (28) were obtained from their main caregivers, while SMFQ (29) and APSS (30) were from the children at both waves.

Environmental Factor Assessment

Environmental factors such as bullying/bullied involvement and warm parenting style were obtained from the caregivers. In addition, bullying/bullied involvement was also assessed by the children themselves. Parental depressive symptoms were assessed using the Kessler psychological distress scale (K6) (31) at wave 1 and the 28-item version of GHQ-28 (32) at wave 2. Socio-economic status at the children's age 10 were indicated by their annual household income and parental educational attainments from the parental questionnaire (25). Life satisfaction consisted of four items reported by the children at both waves.

Statistical Analysis

The psychological assessment of each survey was tested by an exploratory bifactor analysis using 'psych' package (33) of R version 3.4.4 (34). A bifactor model has a bi-factor structure, composed of a general factor and several group factors, which

can provide a common and specific psychological symptom structure for the dataset (35). We applied a bi-quartimin rotation (36) to fix the correlations between the general factor with the group factors at zero but at the same time allowed non-zero correlations between the group factors. Numbers of factors were determined using a scree test (37). The final number of factors to be retained depended on whether the factors have at least three items with 0.40 or higher factor loadings. After we determined the number of factors, we further deleted items with small factor loadings to get a good fit while allowing some factor loadings to have smaller than 0.40 in the same factor structure.

Confirmatory factor analysis was performed using 'lavaan' package (38). The model was estimated by the maximum likelihood method and the missing values were handled by full information maximum likelihood. The criteria of a fitted model were defined by $p \geq 0.05$ in a chi-square test, or root mean square error of approximation (RMSEA) ≤ 0.05 and comparative fit index (CFI) ≥ 0.90. For the environmental factors, we used exploratory factor analysis followed by confirmatory factor analysis, with the same steps.

Finally, SEM using 'lavaan' package was conducted to explore the associations and relationships between environmental factors and psychological symptoms from wave 1 to wave 2 in one model. The paths of relationships were set under time course. Non-significant paths ($p > 0.1$) were removed, and covariance was set to zero. We calculated the models until the coefficient of every path and covariance's p value become less than 0.05.

RESULTS

Demographic Characteristics

All psychological symptom scale scores decreased in 2 years except for the peer relationship sub score in the SDQ and the APSS score (p's < 0.05, **Table 1**). Similarly, bullying/bullied involvement, except for "taking others' things", decreased significantly. Boys at age 10 had greater psychological symptoms in the SDQ, SMFQ, and APSS scores compared to girls, but such difference became non-significant in some scores at age 12 (**Tables S1** and **S2**). Similar trends of gender difference were seen in bullying/bullied involvement, but girls had greater bullying/bullied involvement for 'left out' compared to boys at age 12. Most of the correlations between psychological symptoms were significant in both waves (**Figure S1**).

Bifactor Models for Psychological Symptoms

For psychological symptoms, data from the two waves both suggested a five-factor structure that comprised a general psychopathology factor and four unique factors (depressive symptoms, aggressive behaviors, psychotic symptoms, and somatic symptoms; age 10: $\chi^2 = 787.5$, $df = 150$, $p < 0.001$, $CFI = 0.953$, $RMSEA = 0.037$, **Figure 1A**, **S2A** and **Table S3**; age 12: $\chi^2 = 639.6$, $df = 150$, $p < 0.001$, $CFI = 0.962$, $RMSEA = 0.033$; **Figure 1B**, **S2B** and **Table S4**).

The models consisted of 20 items at both ages; 15 items were common in both models (**Tables S3** and **S4**, respectively). Six items were specific to general psychopathology related to the internalized problems of anxiety from the CBCL and SDQ. Depressive symptoms, aggressive behaviors, psychotic symptoms, and somatic symptoms consisted of five items from the SMFQ, three items from the CBCL and SDQ, three items from the APSS, and three items from the CBCL and SDQ, respectively.

Factor Analyses for Environmental Factors

Wave 1 data appeared to support a six-factor structure which consisted of the child's life satisfaction, bullied, bullying, parental depressive symptoms, warm parenting style, and socioeconomic

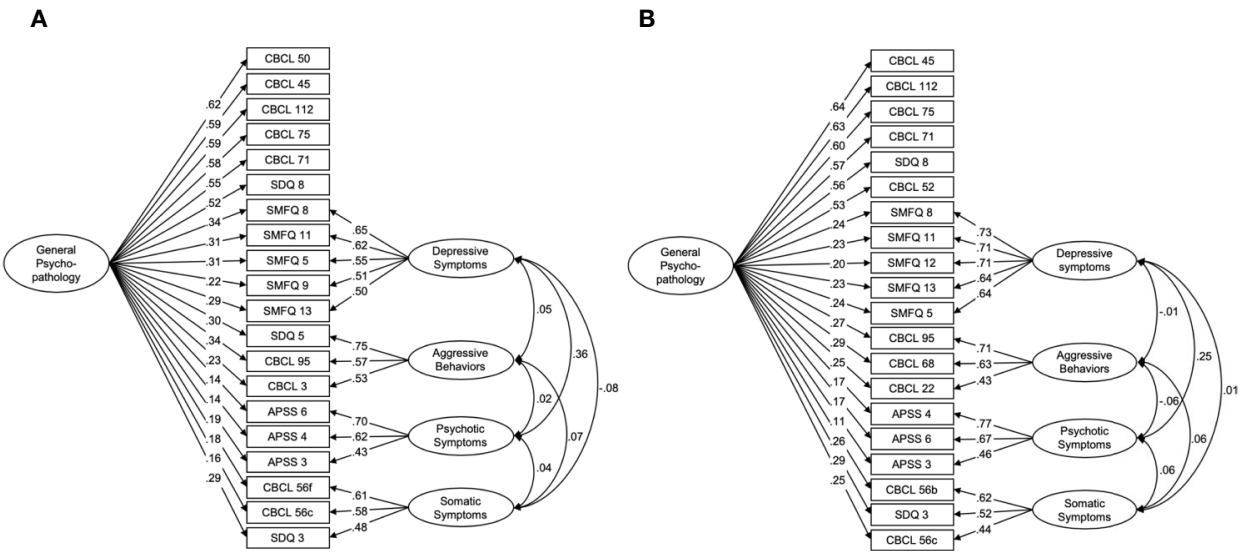

FIGURE 1 | Bifactor models for psychological symptoms at ages 10 and 12. The best fit models at ages 10 **(A)** and 12 **(B)** include unique factors of depressive symptoms, aggressive behaviors, psychotic symptoms, and somatic symptoms, as well as a common factor of general psychopathology. CBCL, the child behavior checklist; SDQ, the strength and difficulties questionnaire; SMFQ, the short mood and feeling questionnaire; APSS, the adolescent psychotic-like symptom screener.

status (χ^2 = 800.8, *df* = 194, $p < 0.001$, *CFI* = 0.963, *RMSEA* = 0.031; **Figure S3A** and **Table S5**). Wave 2 data supported a five-factor structure that contained the same latent variables as wave 1 except the socio-economic status factor (χ^2 = 844.6, *df* = 192, $p < 0.001$, *CFI* = 0.960, *RMSEA* = 0.041; **Figure S3B** and **Table S6**).

Relationships Between Psychological Symptoms and Environmental Factors in One Model

The latent structures of the psychological symptoms (bi-factor models) and the environmental factors had a good fit in a SEM model (χ^2 = 10371.94, *df* = 3292, $p < 0.001$, *CFI* = 0.907, *RMSEA* = 0.026; **Figures 2–4**). Each symptom and environmental factor at age 12 was mostly influenced by the correspondent symptom and factor at age 10 (β = 0.14 ~ 0.89, *p's* < 0.01; **Figure 4**).

Some correlations between factors were significant at age 10 but became non-significant at age 12 (**Figures 2** and **3**): symptom-symptom correlations between depressive symptoms and aggressive behaviors (β = 0.17, *SE* = 0.02, $p < 0.01$ at age 10, the same below), aggressive behaviors and somatic symptoms (β = 0.11, *SE* = 0.03, $p < 0.01$), and psychotic symptoms and somatic symptoms (β = 0.06, *SE* = 0.03, p = 0.03); symptom-environmental correlations between general psychopathology and warm parenting style (β = -0.10, *SE* = 0.02, $p < 0.01$), depressive symptoms and parental depressive symptoms (β = 0.06, *SE* = 0.02, $p < 0.01$), aggressive behaviors and life satisfaction (β = -0.11, *SE* = 0.02, $p < 0.01$), bullied (β = 0.13, *SE* = 0.02, $p < 0.01$), bullying (β = 0.13, *SE* = 0.03, $p < 0.01$), psychotic symptoms and parental depressive symptoms (β = 0.06, *SE* = 0.02, p = 0.02), somatic symptoms and life satisfaction (β = -0.07, *SE* = 0.02, $p < 0.01$), bullying (β = 0.05, *SE* = 0.03, p = 0.04), and parental depressive symptoms (β = 0.09, *SE* = 0.02, $p < 0.01$); environmental-environmental correlations between life satisfaction and parent depressive symptoms (β = 0.05, *SE* = 0.03, p = 0.04), bullied and parent depressive symptoms (β = 0.10, *SE* = 0.02, $p < 0.01$) and warm parenting style (β = -0.06, *SE* = 0.02, $p < 0.01$), bullying and parent depressive symptoms (β = 0.06, *SE* = 0.02, p = 0.01), warm parenting style (β = -0.06, *SE* = 0.02, p = 0.02), and parent depressive symptoms and warm parenting style (β = -0.09, *SE* = 0.02, $p < 0.01$).

The inter-factor relationships between the psychological symptoms showed that general psychopathology at age 10 was negatively associated with depressive and somatic symptoms at age 12 (β = -0.17, *SE* = 0.05, $p < 0.01$; β = -0.18, *SE* = 0.06, $p < 0.01$; respectively). Psychotic and somatic symptoms were associated with each other (psychotic to somatic: β = 0.10, *SE* = 0.03, $p < 0.01$; somatic to psychotic: β = 0.07, *SE* = 0.03, p = 0.01). Somatic symptom at age 10 was negatively associated with aggressive behaviors at age 12 (β = -0.08, *SE* = 0.03, $p < 0.01$).

The relationship between environmental factors and psychological symptoms revealed that parental depressive

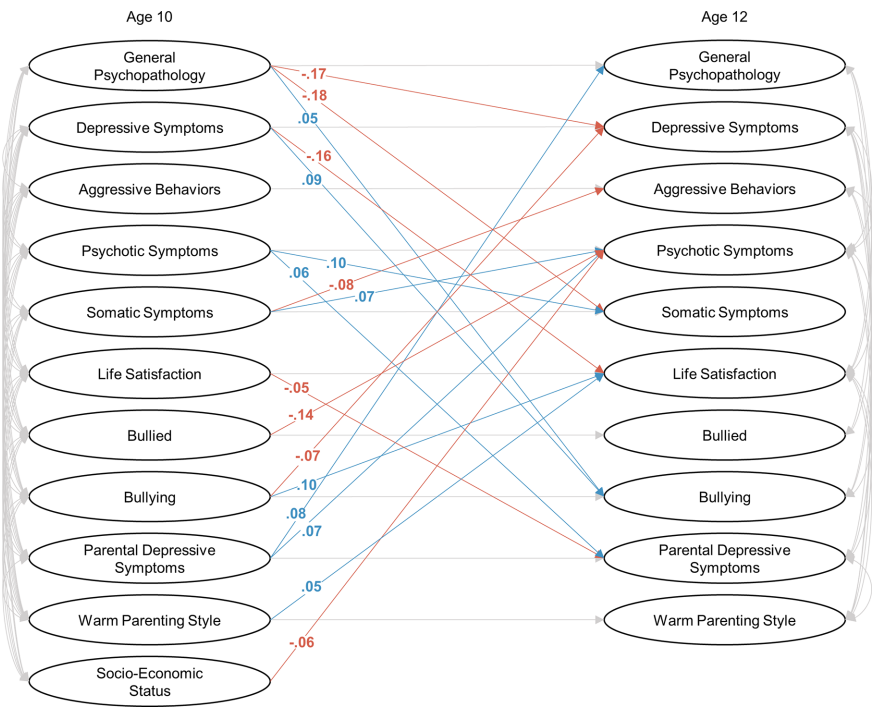

FIGURE 2 | Bifactor models combining with a structural equation modeling at ages 10 and 12. Significant positive and negative relationships between one and another factor were shown in blue and red, respectively. The correlations and relationships in one factor between ages 10 and 12 were shown in gray, and standardized coefficients were shown in **Figures 3** and **4**.

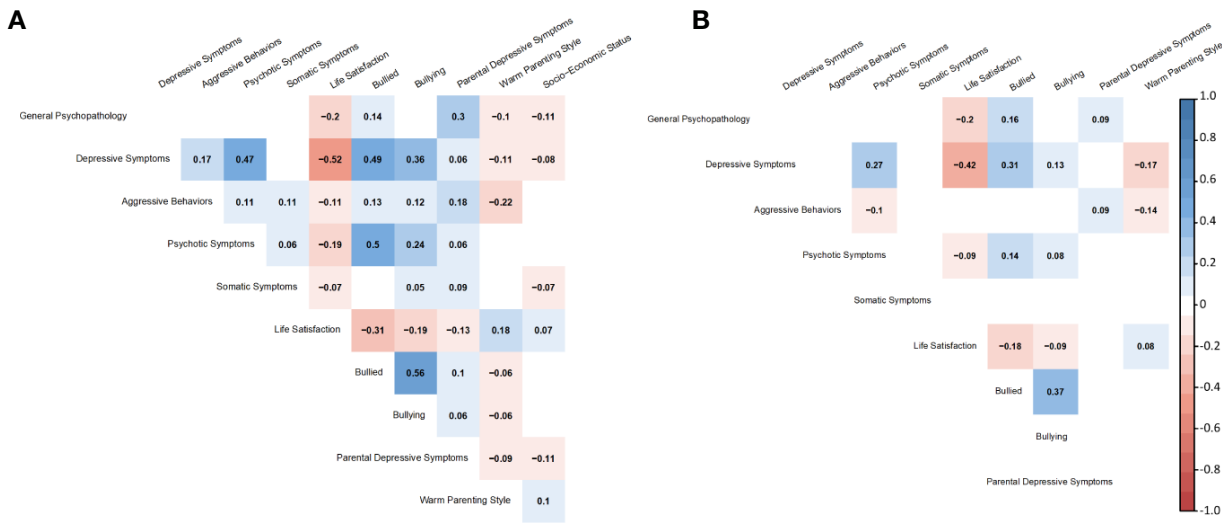

FIGURE 3 | Standardized coefficients of correlations between latent variables at ages 10 **(A)** and 12 **(B)**.

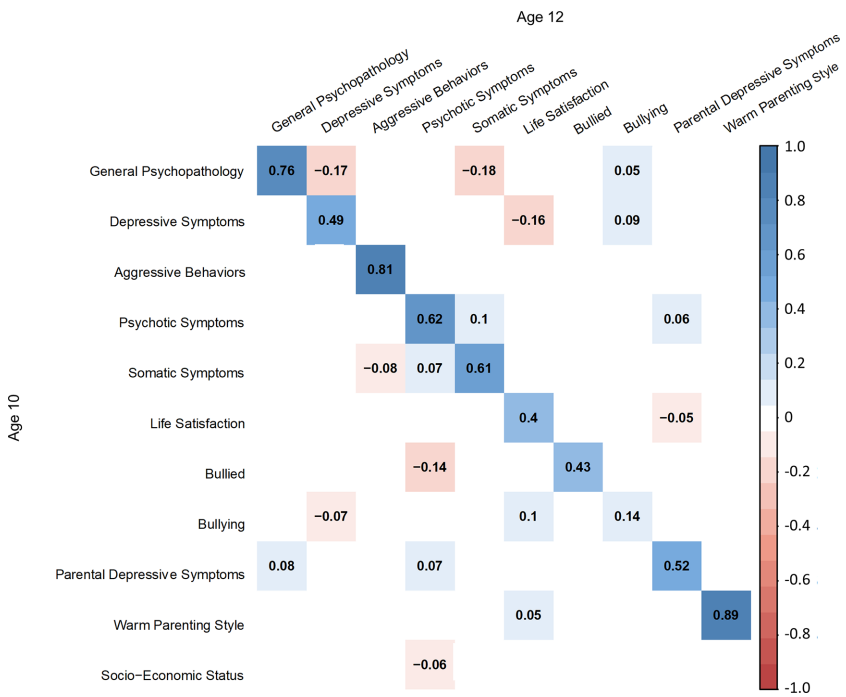

FIGURE 4 | Standardized coefficients of regression between latent variables at ages 10 and 12.

symptom at children's age 10 was positively associated with general psychopathology (β = 0.08, SE = 0.02, $p < 0.01$) and psychotic symptom at age 12 (β = 0.07, SE = 0.02, $p < 0.01$). Bullying at age 10 was negatively associated with bullied with psychotic symptom (β = -0.14, SE = 0.03, $p < 0.01$) and depressive symptom at age 12 (β = -0.07, SE = 0.03, $p < 0.01$). Lower socio-economic status was associated with psychotic symptoms at age 12 (β = -0.06, SE = 0.03, $p < 0.01$).

For symptom to environment paths, life satisfaction at age 12 was affected by depressive symptom (β = -0.16, SE = 0.03, p < 0.01), parenting style (β = 0.05, SE = 0.02, p = 0.01), and bullying (β = 0.10, SE = 0.03, p < 0.01) at age 10. Bullying at age 12 was also affected by general psychopathology (β = 0.05, SE = 0.03, p = 0.04) and depressive symptom (β = 0.09, SE = 0.03, p < 0.01) at age 10. Parental depressive symptoms at age 12 was affected by psychotic symptom (β = 0.06, SE = 0.02, p = 0.01) and life satisfaction (β = -0.05, SE = 0.02, p = 0.01) at age 10.

DISCUSSION

In this study, we investigated the relationships between multiple psychological symptoms and environmental factors simultaneously using a bifactor model in combination with an SEM approach in a population-based cohort from childhood to early adolescence. Apart from the positive relationships between psychological symptoms and environmental factors at the same waves, there were bi-directional relationships between them. Especially, we found a relationship between general psychopathology at age 10 and bullying behavior at age 12, and between parental depressive symptoms at age 10 and general psychopathology at age 12. Unexpected results were also seen, for instance, bullying/bullied involvement was negatively associated with later symptoms. To the best of our knowledge, this is the first study that showed the bi-directional relationships between multiple psychological symptoms and environmental factors in one statistical model.

In line with previous studies, we found significant correlations between psychological symptoms and environmental factors at both waves. Comparing the two waves, correlation coefficients between symptoms and environmental factors decreased overall and some correlations became non-significant from age 10 to 12. The reduction in correlations could be due to an increase of specific psychopathologies which may become more differentiable from other symptoms. In addition, the child-parent relationship could change during this period (39) and parental response pattern for child behaviors and symptoms may also change. Parental responses could be influenced by parental mental state and subjective evaluation of their children, leading to an over/under-evaluation. A decrease in correlations was also seen among environmental factors. For example, bully involvement was correlated with parental depressive symptoms and parenting style at children's age 10, while the correlations became non-significant at 12. This may be caused by a shift of children's significant other from parents to peer, and hence the child-parent relationship becomes less influential (40).

After examining the correlations between psychological symptoms and environmental factors at each wave, we found that general psychopathology at age 10 related to bullying behavior at age 12 and parental depressive symptoms at age 10 related to general psychopathology at age 12. According to the comparative studies between Japan and England, Japanese involvement in bullying tended to happen more in "friends' group" and by more numbers of bullying persons (41). Bifactor analyses showed that general psychopathology factor in this study mainly consisted of anxiety-related symptoms from parental questionnaires at both waves, and therefore, it is possible that highly anxious students involve more in bullying because they are anxious about being bullied themselves. Also, the influence of parental depressive symptom to child's mental states is consistent with previous literature (13, 14). Our results strengthen the view that close attention should be paid to parental mental state in order to prevent the development of general psychopathology in children. Still, another possible explanation for this finding is that parental mental state may influence their recognition of the value in their children. Future studies should use more reliable evaluation of anxiety and depression in the combination of psychological interviews and multi-dimensional reports (i.e. child, parents, and teachers).

Unexpectedly, we found that bullying/bullied involvement was negatively associated with later psychological symptoms, which was contrary to our initial hypothesis. A number of studies revealed that bullying/bullied involvement has a strong impact on future emergence of psychological symptoms and psychiatric disorders (5–7, 17, 42, 43). One possible explanation for this finding may be that the awareness and experience of bullying/bullied in their primary schools may rather support the decrease of psychological symptoms in their junior high schools. During the waves, the participants moved into junior high schools, and therefore, their school environment and peer relationship changed dramatically. This awareness and experiences may have strengthened their psychological resilience toward later symptom emergence.

A decrease in the number of children reported bullied, bullying and psychological symptoms from age 10 to age 12 has frequently been reported, and the former trend was also observed in our study (44–46). A study shared the same target age group with us and is an exceptional case where such a pattern was not observed (47). They have found a positive bidirectional relationship between bullied experience, and depression and anxiety at age 10–11 and 12–13 using path analysis. This may suggest that the change in our sample's response pattern to the instruments contributes to the unexpected correlations found. Another possibility is that our data was obtained from parental responses for anxiety-related items and both parental and children's responses in depression and bully-related items. The multi-informant assessment generally increases the validity and reliability of the children's responses (48), but it would reduce the tendency of positive response. Since early adolescence is a critical period to the validity and reliability of the responses, results obtained regarding the psychological symptoms and bully involvement could considerably change according to who responded the information, future studies considering this will be needed. Compared to this study, pervious research on the impact of bullying/bullied involvement to later psychological symptoms was based on a longer observation period through adolescence (5, 43) and adulthood (6, 7, 17, 42). To investigate the long-term impact of bullying/bullied involvement, we plan to follow-up and obtain data at age 14 and later in TTC. We also plan to perform a stratified analysis to see how each factor changes across adolescent development and further test the relationship

between psychological symptoms and environmental factors. It would be interesting to investigate the critical time period or duration of bully involvement, which could lead to development in psychiatric disorders in further longitudinal studies.

There are some limitations in this study. First, as discussed above, we distinguished specific symptoms from general psychopathology; however, there could be response bias included in the general psychopathology factor (49, 50). In this study, parental responses applied to the models more than children's responses, and the general psychopathology factor could be biased by parental mental condition and response pattern. Second, adolescent physical and psychological development may alter the understandings of items in questionnaires and thus change their responses. Third, a considerable change in physical and psychological development according to the beginning of secondary sex characteristics could alter the pattern of psychological symptoms and environmental factors (51). Gender differences were not specifically investigated in the model since the sample size for each gender is still too small to converge and fit the model. In previous studies, there were relatively small gender differences in symptoms and environmental factors compared to those for adolescence and adulthood. Fourth, as bifactor model approach required a considerable amount of variance in responses, some rare but crucial psychological and environmental items were dropped from model construction such as criminality (52), severe bullying/bullied involvement (7), and abuse (16, 53). Multi-dimensional responses for assessing the severity of the psychological symptoms and risk factors and relatively lower prevalence of them may provide more reliable identification of the cases (54, 55). Thus, further investigations are needed to build a more robust model by combining the statistical approach with more reliable responses.

The present study showed the bi-directional relationships between multiple psychological symptoms and environmental factors from childhood to early adolescence in one model. The correlations and relationships were mostly consistent with previous studies; however, some contradictory relationships to previous findings were also seen. The reason could be partially explained by the change of response pattern for both children and their parents according to adolescent development and their socio-environmental changes in this period. Bifactor modeling combining with an SEM approach enabled us to figure out the unique relationships between psychological symptoms and environmental factors. The results provide a better understanding of the emergence of psychological symptoms and the relationships with environmental factors from childhood to early adolescence.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: Data from TTC is archived in the Tokyo Metropolitan Institute of Medical Science. Collaboration in data analysis and publication will be welcome through specific research proposals sent to the research committee. The initial contact point for collaborations is [nishida-at@igakuken.or.jp]. Requests to access these datasets should be directed to AN, nishida-at@igakuken.or.jp.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of Tokyo Metropolitan Institute of Medical Science (number: 12-35), the University of Tokyo (number: 10057), and SOKENDAI (the Graduate University for Advanced Studies, number: 2012002). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

ZH and SK designed the work, conducted statistical analyses, and wrote the draft manuscript. KE, SY, SF, SA, AN, and SK contributed to data acquisition and managed the quality of the dataset. 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/fpsyt.2020.574182/full#supplementary-material

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Anxiety, Reinforcement Sensitivity and Social Context in Accepting the Experience of Pain Among Rheumatoid Arthritis Patients

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Background: Acceptance has become one of the most widely studied processes regarding chronic pain because of its ability to influence participants' adaptation and coping responses. Leading researchers have found relationships between variables such as anxiety, reinforcement sensitivity, and the responses of the participants' environment to their behavior and acceptance. In contrast, few studies have been found that investigate the variables that predict the acceptance of pain. This study has set out to explore the relationships between pain-related anxiety, sensitivity to contingencies, and the punishment responses of significant people toward pain behaviors regarding pain acceptance.

Methods: With a view to fulfilling this purpose, a cohort of 62 participants with rheumatoid arthritis was chosen, and the subjects were assessed through the following self-report measures: Chronic Pain Acceptance Questionnaire, CPAQ; Pain Anxiety Symptoms Scale, PASS-20; The Sensitivity to Punishment and Sensitivity to Reward Questionnaire, SPSRQ, and The West Haven-Yale Multidimensional Pain Inventory, WHYMPI. The study's initial objectives were achieved by means of a stepwise multiple linear regression analysis.

Results: The linear regression analyses revealed a negative and significant correlation between anxiety, reinforcement sensitivity, and the significant persons' responses to pain behaviors and pain acceptance.

Conclusion: The results suggest that the identification of these variables might be important for addressing these participants' pain. Finally, the discussion focuses on our findings' implications as regards their use in clinical practice.

Keywords: mindfulness, cognitive behavioral therapy, social influences, reinforcement sensitivity, anxiety, acceptance, chronic pain

INTRODUCTION

Chronic pain has latterly become an increasingly serious health issue, as reflected by the new steps taken by the World Health Organization, which has now classified this experience as a major illness (1). The foremost epidemiological studies indicate that chronic pain is highly prevalent worldwide (2–4). Its most serious consequences include impacting upon the quality of life of participants and their families, negative psychological effects, the disability it causes, problems associated with the loss of productivity at work, and the high socio-economic costs incurred by the health system (5–9). Therefore, and in view of these circumstances, it is understandable that chronic pain is now considered a major public health issue (10), becoming a key study topic for leading researchers in the field.

Acceptance has been one of the more widely studied pain-related variables in recent years, as it has rapidly become a significant process for the applied clinical context because of its ability to influence the manner in which participants adapt to and cope with the experience of responding to pain (11–17). On a theoretical level, we are dealing with a complex construct that scholars have interpreted differently (16). Nevertheless, the acceptance of pain has traditionally been defined as an individual's constant readiness to experience pain (thoughts, feelings, sensations, etc.) without offering any resistance, while channeling their behavior toward valuable goals and objectives (11). According to McCracken (16), this definition has two vital components for its understanding: openness or receptivity to pain, and involvement in activities. The component of openness means surrendering to sensorial aspects, to pain-related feelings, thoughts, and emotions. The second component of involvement refers to a subject's commitment to tailor their behavior according to their values and continue with their everyday activities despite the pain. Based on this conceptualization as our reference framework, we have found numerous studies that relate the acceptance of pain to more adaptive coping, being associated with a lower emotional, physical, and social function (11, 13, 18–22), a lower level of reported pain (23–25), less disability (26), and a reduced use of medication (12). Along these same lines, we find solid evidence to show how clinical interventions based on the acceptance of pain, such as acceptance and commitment therapy (ACT) (27) or mindfulness-based interventions (MBIs) (28), are more effective than processes already in place (29–34). The data available have shown that acceptance is a highly important variable in chronic pain, both at theoretical level and in the field of applied clinical treatment; nevertheless, we have found very few studies that have addressed the psychological variables that might predict higher levels of pain acceptance. Finding these predictors will help to improve the process of selecting the treatment to be followed with these participants.

Anxiety has been described in the literature as a significant factor in acceptance processes in different samples of participants with chronic pain. High levels of pain-related anxiety have therefore been associated with lower levels of openness toward the same, and less involvement in activities by the sufferer; in other words, there seems to be a strong, negative relationship with acceptance (11–13, 21, 35–37). Elsewhere, we encounter studies that address anxiety sensitivity (AS), which has been defined as a trait that predisposes someone to experience a fear of pain and develop anxiety disorders (38). Several scholars have posited that through its predisposition to the fear of pain, AS is directly related to the adoption of escape or avoidance behaviors (39–41). Experiential avoidance is a key pattern of behavior that is located at the other extreme from acceptance (42), whereby it may be argued that AS is indirectly related to the acceptance of this feeling. When we consider the findings of these studies as a whole, they all suggest that anxiety plays a crucial role as a predictor of low acceptance in contexts of chronic pain.

In addition, and in this same vein, there are two known neuropsychological systems that can impact upon avoidance and approach behaviors: the Behavioral Approach/Activation System (BAS) and the Behavioral Inhibition System (BIS). The most widely cited theory of the different approach-avoidance models is Reinforcement Sensitivity Theory (43, 44). This model has recently been reviewed within the field of chronic pain [to read the review, see (45)]. This model indicates that the guidelines for behavioral approach or avoidance in certain situations depend on contextual keys (internal or external), which predict the probability of receiving a reward or a punishment (46). The BAS is therefore triggered by the presence of keys that indicate the possibility of obtaining a reward, or of eliminating or reducing the likelihood of an aversive stimulus, while the BIS is triggered by the presence of keys that predict a punishment (e.g., pain, disability, catastrophic thoughts, and anxiety). Numerous researchers have found that participants with chronic pain record more BIS activity and less of BAS (47–51). These systems are in some way mutually inhibited, and their alternance can be explained by sensitivity in the presence of the aversive or appetitive stimulus (52). We have found certain studies that report that these participants are more sensitive to reinforcement than control groups (47, 53). An analysis of this information is expected to show that sensitivity to reinforcement and punishment is related to the adoption of behaviors of greater or lesser openness and involvement regarding pain, and therefore to its acceptance. Furthermore, sensitivity to punishment is also associated with less social activity and a lower probability of social support (54), with the latter being a highly important variable in coping with chronic pain (55, 56).

Related to this last point, research has focused its attention on interpersonal relationships involving participants with chronic pain, and more specifically within the family setting. According to the theory of operant conditioning, the immediate environment's response has the ability to promote behaviors

Abbreviations: ACT, Acceptance and Commitment Therapy; AMAPAR, Association of Participants with Rheumatoid Arthritis; AS, Anxiety Sensitivity; BAS, Behavioral Approach/Activation System; BIS, Behavioral Inhibition System; CBT, Cognitive Behavioral Therapy; CPAQ, Chronic Pain Acceptance Questionnaire; MBIs, Mindfulness-based interventions; PASS-20, Pain Anxiety Symptoms Scale; SP, Sensitivity to punishment; SPSRQ, The Sensitivity to Punishment and Sensitivity to Reward Questionnaire; STR, Sensitivity to reward; WHO, World Health Organization; WHYMPI, The West Haven-Yale Multidimensional Pain Inventory.

of pain or well-being among participants with chronic pain (57). Many studies have reported that solicitous responses (e.g., expressions of support or concern, or instrumental support for the pain behavior) and punishing responses (e.g., expressions of frustration or irritation toward the pain behavior) by significant people close to the patient are linked to an increase in pain, lower levels of activity, more pain behaviors, more visits to the doctor, and greater disability (58–67). This means that significant people's reaction to these participants' pain behaviors may have an indirect impact on pain acceptance processes. Furthermore, relatively large studies involving participants with chronic pain have found a strong and negative relationship between solicitous and punishing responses and pain acceptance (68), maintaining its predictor value even a year after the medical intervention (69).

The information provided as theoretical underpinnings has informed this study designed to examine the relationship between pain-related anxiety, sensitivity toward punishment and reinforcement, significant people's response to pain behaviors, and its predictive capacity in terms of pain acceptance, due to the relationship shown by these variables in the aforementioned studies. Results will inform treatment decision-making and the standard of psychological care provided to people with chronic pain.

METHODS

Participants

The study was approved by the Research and Ethics Committee of CEIC Hospital Clínico San Carlos in Spain. Subjects eligible for the study were patients with rheumatoid arthritis ($n = 62$) participants who were undergoing treatment in the Department of Rheumatology at the hospital and at the Madrid Association of Participants with Rheumatoid Arthritis (AMAPAR, in its Spanish acronym). All the data required for the study were gathered between December 2015 and February 2017. Subjects were screened by phone about their interest of participation in the study, only participants with higher interest were selected for evaluation. Subjects who indicated that they were medically healthy, other than rheumatoid arthritis, aged $\geq$18 years willingness to give consent and participate in the study, were asked to meet the lead researcher on a face-to-face interview for individual assessment. The assessment was conducted individually in a single session by the same assessor, without any limit of time. On average, each session took one and a half hours. During the evaluation process participants were excluded if they had: (1) a history of psychiatric disorder such as major depressive disorder, obsessive-compulsive disorder or anxiety generalized disorder schizophrenia; (2) lack of motivation to complete the self-report measures; (3) or high levels of alcohol/substance abuse. Female patients who were pregnant or lactating women were not grounds for exclusion. The participants who refused to complete data on all self-report measures listed below were excluded from final sample ($n = 6$). Participant characteristics are presented in a table in results section (see **Table 1**). The study design is presented in **Figure 1**.

TABLE 1 | Sociodemographic characteristics and clinical variables of the study participants.

Characteristic	Frequency (*n*)	Percentage (%)
Sex		
Female	13	21
Male	49	79
Age in years *(M, SD)*	53.2 ± (11.2)	
Marital status		
Single	15	24.2
Married	31	50.0
Widowed	3	4.8
Divorced	7	11.3
Separated	6	9.7
Education level		
Primary	7	11.3
EGB or equivalent	7	11.3
Technical and vocational	10	16.1
Senior high school	17	27.4
University	15	24.2
Higher education	3	4.8
Unregulated studies	3	4.8
Socioeconomic status		
Low	13	21
Medium	43	69.3
High	6	9.7
Time elapsed since the first medical diagnosis		
Less than a year	4	6.4
Less than 3 years	4	6.5
Less than 5 years	2	3.2
Between 5 and 10 years	15	24.2
More than 10 years	37	59.7
Pharmacological treatment		
None	1	1.6
Biological agents (e.g., Infliximab, Abatacept, etc.).	2	3.2
FAMES (e.g., Metotrexato).	1	1.6
Corticosteroids	0	0
Anti-inflammatory drugs	1	1.6
Analgesic drugs	0	0
Others	2	3.2
Several of the above	55	88.7

M, mean; SD, standard deviation.

Ethical Statement

The study protocol was approved by the Research and Ethics Committee of CEIC Hospital Clínico San Carlos in Spain, registration number 15.531-E. Date of communication: 10 December 2015. All participants signed a consent form after been informed about eligibility criteria and study procedures. The lead investigator monitored the data collection and informed consent process. Only participants who completed data for all self-report measures listed below were included in the sample and taken into consideration for the statistical analyses. Those who

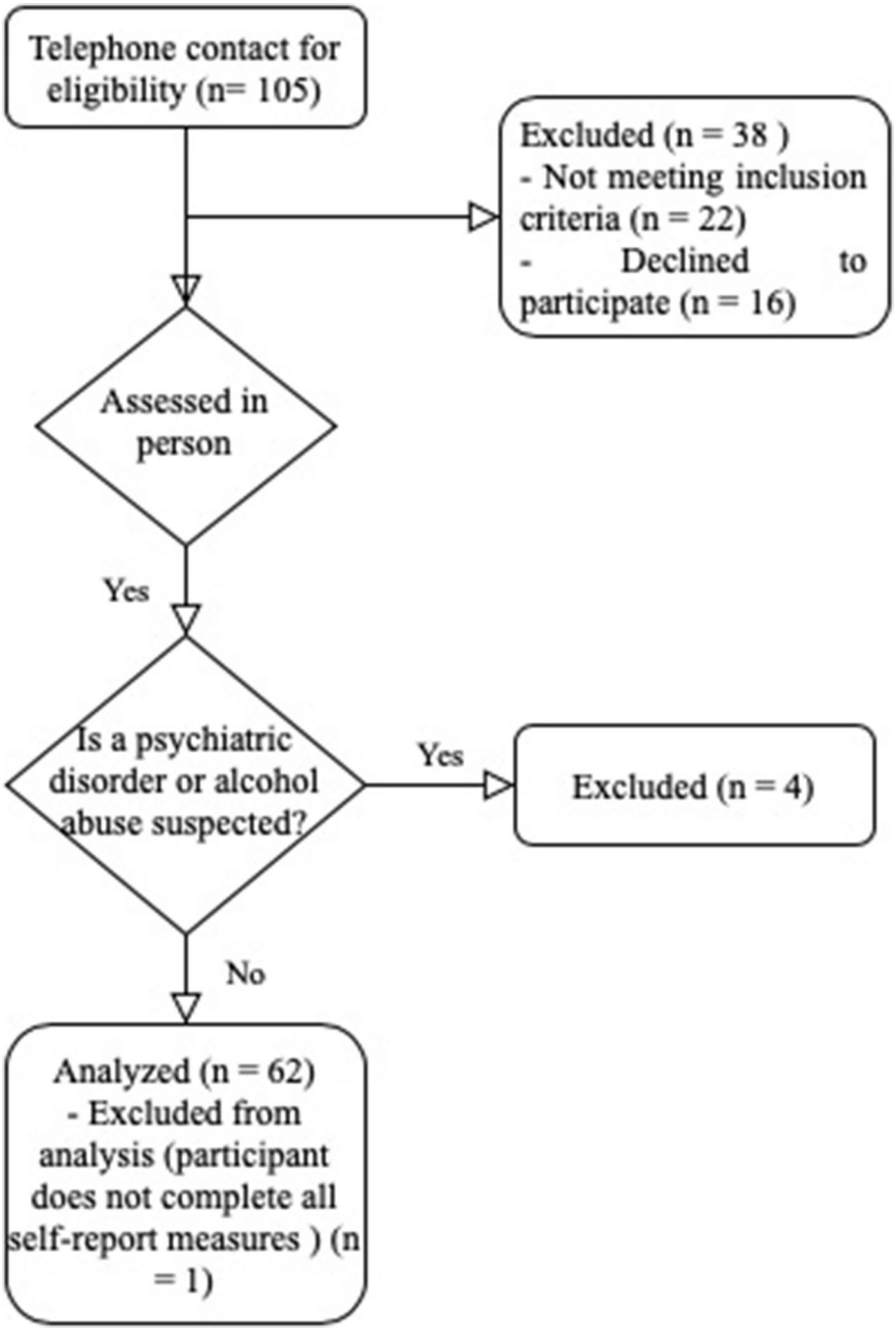

FIGURE 1 | Study design flowchart.

did not meet the conditions of eligibility previously mentioned were discarded.

Procedure and Self-Report Measures

The self-report measures were administered on a face-to-face basis at the Department of Rheumatology at the Hospital Clínico San Carlos in Madrid (Spain) and at AMAPAR. Only five participants were assessed by videoconferencing via Skype. The participants were invited to take part in the study, and once they had voluntarily agreed to do so, they left their phone number for the initial contact. The lead researcher subsequently contacted the interested parties to give them information on the study and arrange a meeting. The assessment was conducted individually in a single session, without any limit of time, and always involved the same assessor. During the appointment, the participants signed the informed consent form and completed a

socio-demographic questionnaire, answering questions about the nature of the pain, the time elapsed since their first diagnosis, and the medical treatment they were following. Finally, they also completed a series of self-report measures on the psychological variables to be studied, as described in what follows.

- **CPAQ**. *Chronic Pain Acceptance Questionnaire* [(16): Spanish version by Menéndez (70)]. It consists of 20 items that assess the acceptance of pain in participants with chronic pain. The questionnaire has two subscales: openness to pain and involvement in activities. The former refers to an individual's willingness to experience pain without putting up any resistance, while the latter assesses an individual's ability to take part in activities despite the pain. The answers involve a Likert-type scale from 0 (never true) to 6 (always true). Our sample recorded suitable levels of internal consistency reliability for the total scale (Cronbach's alpha = 0.690), according to the criteria proposed by Prieto (71).
- **PASS-20**. *Pain Anxiety Symptoms Scale* (72). This scale explores anxiety responses to pain: fear, escape/avoidance, physiological anxiety, and cognitive anxiety. It consists of 20 items with Likert-type answers ranging from 1 (never) to 5 (always). Our sample has recorded suitable criteria for internal consistency reliability through Cronbach's alpha coefficient (presented in brackets) for its five component subscales: fear (0.772), escape/avoidance (0.649), physiological anxiety (0.598), cognitive anxiety (0.811), and overall scale (0.880). They are suitable according to Prieto's criteria (71).
- **SPSRQ**. *The Sensitivity to Punishment and Sensitivity to Reward Questionnaire* (73). This is a self-report measure consisting of 48 items with a dichotomous (Yes/No) answer format. It is divided into two subscales, each with 24 items: sensitivity to reward (STR) (behaviors focusing on the search for reinforcers, such as the search for sensations, money, or power), and sensitivity to punishment (SP) (behaviors designed to avoid aversive stimuli or negative consequences, due to the possibility of harm or failure). Through Cronbach's alpha coefficient (presented in brackets), this study has recorded acceptable levels of reliability for STR (0.725) and good ones for SP (0.825), which were appropriate according to Prieto's criteria (71).
- **WHYMPI**. *The West Haven-Yale Multidimensional Pain Inventory* [(74); Spanish version by Ferrer (75)]. The study applied the second domain of the questionnaire corresponding to the subscale that assesses the reinforcing and punishing responses provided by the caregivers in response to a patient's pain behaviors. This section consists of 14 items with a Likert-type response format ranging from 0 (never) to 6 (very often). The measure has recorded good levels of reliability in our sample (Cronbach's alpha = 0.842) according to the criteria proposed by Prieto (71).

Data Analysis

The data were coded and analyzed using version 25.0 of the SPSS statistical package. The goals considered here involved conducting multiple stepwise linear regression exploratory analyses. The predictor variables used were cognitive anxiety, reinforcement sensitivity, sensitivity to punishment, the reinforcements and punishments administered by the patient's carers in response to pain behaviors, as well as the variables to be controlled (age, time elapsed in months since the first symptoms of pain, socioeconomic status, and the current medical treatment being received). The acceptance of pain was used as the dependent variable or criterion variable. A series of prior tests were carried out to ensure compliance with the assumptions of normality, revealing a suitable distribution of the residuals. In terms of homoscedasticity, the Durbin-Watson results (1.656) are within the recommended range (2 ± 0.5). The tolerance values for the variables introduced were below 0.10, dismissing any problems of collinearity. These statistics therefore tell us that these data are suitable for a linear regression analysis.

RESULTS

Sample Characteristics

The sample consisted of 62 participants (13M/49F), the mean age was 53.24 (SD = 11.29), ranging between 25 and 77. Half of the participants were married or in a long-term partnership (50%), followed by those that were single (24.2%), divorced (11.3%), separated (9.7%), and finally, widowed (4.8%). Regarding their educational status, many participants had completed primary ($n = 7$, 11, 3%), secondary school ($n = 17$, 27, 4%), or university ($n = 18$, 29%) studies. About a third of them had completed some type of tertiary or vocational education ($n = 10$, 16.1%). Altogether, 69.3% reported medium incomes ($n = 43$, 69.3%). The distribution of the time elapsed since the first medical diagnosis in our sample was less than a year ($n = 4$, 6.4%), <3 years ($n = 4$, 6.5%), <5 years ($n = 2$, 3.2%), between 5 and 10 years ($n = 15$, 24.2%), and more than 10 years ($n = 37$, 59.7%). Most of the participants were following a pharmacological treatment based on FAMES ($n = 1$, 1.6%), anti-inflammatory medication ($n = 1$, 1.6%), biological medication ($n = 2$, 3.2%), others ($n = 2$, 3.2%), and several of these ($n = 55$, 88.7%). Only 1.6% were not receiving any medical treatment. The characteristics of the study participants, based on socio-demographics and relevant clinical variables are summarized in **Table 1**.

Multiple Regression Analysis of the Acceptance of Multiple Regression Analysis With Acceptance as Dependent Variable

With a view to meeting this study's overriding goals of studying the relationships between predictor variables (anxiety toward pain, sensitivity to pain and to reinforcement, and the responses of significant persons to pain behaviors) on the dependent variable (pain acceptance), a multiple stepwise linear regression exploratory analysis has been conducted.

Table 2 shows the results of the correlations between the predictor variables and the acceptance of pain (CPAQ). The analyses revealed a model that added significant persons' punishing responses ($\Delta R^2 = 0.045$) when facing pain behaviors

TABLE 2 | Multiple stepwise linear regression analysis of pain-related anxiety, sensitivity to punishment and reinforcement, and the punishing responses of significant persons toward pain behaviors, on the acceptance of pain*.

Step	Predictors					Regression model				
		B	*β*	*t*	*p*	R	R^2	ΔR^2	*F*	*P*
1	Constant	96.226		18.406	0.00					
	PASS_20_total	−0.681	−0.605***	−5.891	0.00	0.36	0.356	0.366	34.698***	0.000
2	Constant	97.954		19.346	0.00					
	Pass_20_total	−0.548	−0.488***	−4.462	0.00	0.42	0.407	0.060	21.948***	0.000
	Reinforcement sensitivity	−1.098	−0.272*	−2.489	0.016					
3	Constant	99.688		20.083	0.00					
	Pass_20_total	−0.563	−0.501***	−4.722	0.00	0.47	0.444	0.045	17.255***	0.000
	Reinforcement sensitivity	−1.086	−0.269*	−2.541	0.14					
	Whympi punishment	−0.592	−0.212*	−2.222	0.030					

*N = 62. *p < 0.05; ***p < 0.001; B = Non-standardized regression coefficient; Beta = Standardized regression coefficient; PASS_20_Total: Overall score of the pain anxiety symptoms scale; Reinforcement sensitivity: Reinforcement sensitivity subscale of the Sensitivity to Punishment and Sensitivity to Reward Questionnaire; Whympi punishment: Punishment responses to pain behavior in the second domain of the West Haven-Yale Multidimensional Pain Inventory.*

to other variables, such as pain-related anxiety ($\Delta R^2 = 0.060$) and reinforcement sensitivity ($\Delta R^2 = 0.366$). This led to a statistically significant model ($F = 17.255$, $p \leq 0.01$) that explained 44% of the variance on the dependent variable (Adjusted $R^2 = 0.444$). All the correlations that feature in the model were significant when predicting pain-related anxiety, and reached the statistical criterion $p \leq 0.05$ required to do so. The linear regression analyses reveal a negative and significant correlation between the three predictors and the dependent variable, recording an effect size that varies from small to medium ranges according to Cohen's criteria (76).

DISCUSSION

The results presented here reveal that emotional variables such as pain-related anxiety, reinforcement sensitivity, and punishing responses toward pain behaviors by significant people for the patient accurately predict the individual's predisposition to accept pain. As noted, the scope of these relationship has generally been small or moderate. Regression analyses have provided us with a more profound understanding of the relationships between these variables described in the literature.

Pain-related anxiety has proven to be the best predictor of the acceptance of pain. The results are consistent with the findings reported by other scholars on a negative and robust correlation between pain-related anxiety and the components of its acceptance (11–13, 21, 35–37). This therefore highlights the importance that pain-related anxiety might have as a variable linked to the acquisition of fear and escape or avoidance behaviors in the face of pain, as reported by other scholars in the literature reviewed (39–41). According to pain-avoidance models (77, 78), escape behavior impedes an elaborative processing of the stimuli being avoided (e.g., sensorial aspects of pain, thoughts, emotions or sensations) (79), which leads to the acquisition of fear related to the pain itself, and a biased interpretation of the symptoms as threatening (25, 80). This means that if the patient is experiencing high levels of anxiety, it is reasonable to assume that this emotion is going to play an important role in the way the patient suffers and copes with the illness and, therefore, in their clinical treatment.

Our findings show that the STR variable is linked to a greater predisposition toward the acceptance of pain in the presence of higher levels of STR. The results are consistent with the findings reported in other studies, which have noted this variable's importance in participants with chronic pain (47, 53). Nevertheless, prior studies have indicated that participants with chronic pain are expected to have a greater level of activation in the BIS, and a lower one in the BAS, with a greater presence of avoidance behaviors (47–51). Knowing that the activation of both systems is related to SP and STR (81), we expected to find a direct and significant correlation between STR or an indirect correlation between PS and pain acceptance. Nevertheless, these results can be explained when we consider that the perception of reinforcement varies for each person and depends on their psychological state, their values and their goals (82). For example, it is logical that someone with a high STR and greater impulsiveness is more motivated to achieve goals and assign behavioral resources accordingly, although for such a person it might be harder to accept that the pain, or the incapacity associated with it, no longer permits them to do so. It therefore seems probable that this individual may cope by seeking immediate relief for their symptoms in order to resolve the interference in the short term; in other words, the individual will mobilize behavioral resources looking for negative reinforcement, and they are more than likely to record more escape or avoidance behaviors. It therefore seems reasonable to contend that the higher the STR and the greater the impulsiveness, the lower the predisposition to accept pain. Nonetheless, future researchers will be tasked with clarifying this variable's role regarding acceptance and coping in participants with chronic pain.

This study has also uncovered a negative and significant correlation between the punishing responses toward pain behaviors shown by the patient's carers and pain acceptance. These results coincide with other studies that predicted a worse adjustment to pain in the presence of adverse contingencies for the patient (58, 59, 62, 66). The results also coincide with the findings made by McCraken (68), who has reported that the punishing responses of significant people are negatively associated with the acceptance of pain. Therefore, as noted earlier,

the social support of significant persons for pain behaviors seems to be a highly influential variable in acceptance processes in contexts of chronic pain. The paucity of studies on this matter calls for further research designed to extend the information on the relationship between these two variables.

The results forthcoming here prompt us to make a series of suggestions that could help to improve the care provided for these participants. Pain-related anxiety and reinforcement sensitivity are variables to be considered during the assessment process. Whenever high scores are observed in any of these variables, it would be advisable to use some technique (e.g., cognitive restructuring) to work on cognitive aspects or even consider the possibility of a more traditional intervention for correcting a mistaken interpretation of the symptoms, reduce the perception of threat, and boost active coping with the illness, as in Cognitive Behavioral Therapy (CBT), which has proven to be extremely effective in cases of chronic pain [e.g., (83–85)]. In the case of low scores for these variables, the initial choice of treatment could involve any intervention based on third-generation therapies, as both ACT and MBIs have proven to be effective in pain contexts (33, 86–88). The results obtained also refer to the importance of providing families with accurate information on the way patient's behave when dealing with pain and their relationship with the treatment, whereby they can support the patient in a non-interfering manner.

These results and the aforementioned conclusions should be considered within the context of some of their limitations. Firstly, the sample used here involved discarding several participants that did not meet the inclusion criteria, and the final cohort consisted solely of participants with rheumatoid arthritis. Future research should study the relationship between these variables and other groups of participants with chronic pain. Moreover, the final sample is small, particularly in the case of males, so other researchers are advised to employ broader samples in the future with a view to comparing results. It is also important to talk about methodological issues arising from the self-report measure used to assess the main carers' responses to their participants' pain behaviors. This instrument rates the carers' responses based on the individual's own subjective opinion. This perception may be influenced by other psychological variables, which means these data should be interpreted with some caution.

CONCLUSIONS

In sum, variables such as pain-related anxiety, STR, and the punishing responses of significant people for the patient predict a lower acceptance of pain in participants with chronic pain. We may therefore infer the convenience of taking them into consideration during the assessment process in the first clinical contacts. In turn, prior knowledge of these variables may inform the decision-making on the intervention to be performed in each case, which could improve the efficacy or success of this care. Based on the results obtained, there is a need to investigate these variables in relation to the components of the acceptance of pain, given the part they play in the treatment to be followed with these participants.

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 human participants were reviewed and approved by CEIC Hospital Clínico San Carlos. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LP contributed in the conception, design of the study, acquired the data, analyzed and interpreted the patient data, drafted the article and overseed the final version of the article before submission, and was a major contributor in writing the manuscript. MP-N had a relevant role in the conceptualization of the study, analysis and interpretation of the data, and drafting the article. MR contributed in the conception and design of the study. LR-R and LL contributed to acquisition of data. All authors read 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/fpsyt.2020.554990/full#supplementary-material

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The Effect of Subjective Perception of Work in Relation to Occupational and Demographic Factors on the Mental Health of Polish Nurses

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Introduction: Nursing is considered one of the most stressful professions in the world. The high emotional burden associated with excessive workload in qualitative and quantitative terms, exposure to existing and emerging infectious diseases, daily confrontation with the suffering of individuals and their families and low social support leads to the development of numerous stress reactions among nurses, resulting in the development of anxiety, insomnia, social dysfunction and depression. Indeed, somatic and mental stress-related disease rates are higher among nurses than in the general population.

Aim: To determine the impact of subjective work characteristics on the mental health of nurses in relation to demographic and occupational factors.

Material and method: The research was carried out among 558 nurses working in hospitals in Podlaskie Voivodeship, and used the Subjective Work Evaluation Questionnaire (SWEQ) and Goldberg's GHQ-28 Questionnaire.

Results: As measured by SWEQ, and as self-assessed by means of the GHQ-28 questionnaire, *overall stress* negatively affects the nurses' health (R^2 = 18.7%). Among the partial measures of the SWEQ questionnaire, *work overload* had strong and the *lack of rewards, social relations* and *lack of support* had weak negative effect on the overall mental health assessment of nurses (R^2 = 19.2%). The *responsibility* measure was an exception that had a positive impact on the nurses' well-being. Among occupational and demographic factors, only higher education in relation to secondary education in interactions with the *overall stress* measure and *unpleasant work conditions* had a positive effect on the overall mental health self-assessment of nurses (R^2 = 20.7%).

Conclusions: The results of our study provide a clear message to the hospital management that improving the work organization and atmosphere of nurses by reducing perceived work overload and increasing the responsibility of nurses can have a positive impact on their mental health. Encouraging nurses to improve their education

can result not only in an obvious improvement in staff qualifications, but also in better resistance to stressors in the workplace and, consequently, in better staff well-being. Both measures can have a positive impact on the quality of care provided by nurses and on reducing staff turnover.

Keywords: stress, nurse, mental health, anxiety, depression, insomnia, work characteristics

INTRODUCTION

The processes of privatization, automation and globalization in the last 20 years in the Polish health service have resulted in significant changes in the working conditions of medical personnel. Workplaces have become safer in terms of exposure to physical and chemical agents reducing the number of employees falling ill with occupational diseases. Concurrently, the pressure on employee efficiency and cost-cutting has become more prevalent. This yields an increase in the number of employees diagnosed with stress-related diseases on annual basis (1).

Physicochemical and psychosocial factors have negative impact on employees' health through stress. In our study, we focused on the analysis of psychosocial factors. They arise under certain organizational and social conditions and their character is determined by the psychological assessment of their significance to the individual - whether they pose a threat, constraint, deprivation of some important values, or a challenge to his or her abilities and aspirations (2). So far, psychosocial factors have generated relatively little interest among occupational medicine services, primarily due to psychosocial stimuli subjective nature (3). Whether a particular element of a work situation can become a psychosocial stressful stimulus is determined by the characteristics of the individual and the group in which the individual works, since the importance of the factor for the employee depends on individual's work enviroment. Psychosocial factors affect employees' health by triggering long-term stress reactions (2). The effects of these reactions may be reflected in disorders of various systems and body functions.

It is difficult to establish a specific link between a specific psychosocial factor and incidence of a specific disease (4). Hence, in our study we decided to investigate psychosocial factors' effect on the overall mental health of employees. The importance of this issue for research results from several indicators: (1) basically all employees are exposed to psychosocial factors, (2) about 25% of all employees complain about excessive stress at work, (3) trends in the development of work processes have brought about an increase in the number of people employed in the service sector, in managerial positions, in operating computer systems and automated devices, (4) economic effects of stress are seen in the form of reduced work quality, increased number of errors and accidents at work, as well as the costs of treating addictions and stress-related diseases (2, 4).

We decided to conduct our study among nurses. Globally, nursing is considered a very stressful profession (5–8). The high emotional burden associated with increasing workload in qualitative and quantitative terms, exposure to existing and emerging infectious diseases (9), the daily confrontation with the suffering of individuals and their families and low social support leads to many stress reactions among nurses (10, 11). Indeed, studies by Allen and Shanock have shown that as they start their first job, young nurses experience enormous mental shock due to low social support and lack of socialization (12).

Society's expectations toward nurses are significantly different from the feelings of nurses themselves (13). The study conducted by Bolton shows that the expectations of society toward the expression of emotions by nurses concern only those emotions that alleviate the fear and suffering of patients and their families. In contrast, personal emotions, the so-called spontaneous, e.g., frustration, loathing, anxiety, should be expressed with a lot of empathy due to the nature of the performed work and the characteristics of the nursing profession (14). Studies by Donoso et al. indicate that the suppression of emotions so as to reduce emotional expression, e.g., due to working conditions, is a considerable stress factor among nurses (15).

The perception of nurses' workload is a subjective feeling, which has been confirmed by professional stress theories. The theory of individual being fitted to the environment, presented by French et al., is built upon on two basic elements: the degree of attitude and ability of the employee to meet the demands of the job. In this theory, there is a distinction between objective reality and subjective perception of changes taking place in the environment. The objective match is based on external established criteria such as experience, education and skills, which are assessed by external experts, e.g., during job interviews. Subjective fitting refers only to the individual characteristics of the employee and his or her personal perception of the working environment. The mismatch may occur in various configurations, each of which may affect the stress felt by the employee (16).

In studies of occupational or work-related stress, a leading theoretical model that is widely used is the Job Demand Control model developed by Karasek (17). This model predicts that workload and subsequent physical or mental illness are the result of the interaction of work requirements and work control. According to the extended Job Demand Control Support model, the highest risk of mental ill health is expected among employees whose jobs are characterized by high demands, low control and low social support (18).

In opposition, the Siegirist model (19) treats the imbalance between work effort and remuneration. Here, the level of effort depends on two main factors: the characteristics of the work (requirements) and specific personal dispositions. The award for work concerns three aspects: financial gratification and professional status, respect and support, as well as job security and career development opportunities. In a situation where

there is the excessive involvement of an employee in the work performed, with simultaneous underestimation of the prizes received, there is an imbalance between effort and reward, which leads to a stressful situation (20).

The high social expectations and professional demands placed on nurses by hospital management, juxtaposed to the low salaries constitute a perfect match for testing the aforementioned stress theories. Based on numerous studies, it is known that stressors at work have a negative effect on employee health (21, 22). Somatic and mental stress-related disease rates are higher among nurses than in the general population (2). Indeed, such psychosocial burdens affect the development of anxiety, insomnia, excessive sleepiness and depression among nurses (23). The effects of excessive workloads manifest themselves as undesirable behaviors at work such as avoidance, increased irritability and cynical attitude (2). Any workload, which exceeds the employee's ability to cope is associated with absenteeism, change or resignation from work (24). According to Chen et al., some nurses often take sick leave to avoid the mental strain at work and eventually leave the profession (25). In Poland, for example, a significant percentage of nurses leave the profession within 10 years of obtaining their professional qualifications. Here, the main reasons cited for leaving the profession are low wages, difficult working conditions and poor health (26, 27).

Health condition, sickness-related absenteeism and nurses leaving the profession have a direct impact on the quality of care provided and patients' health results (28). We have hence decided to examine which of the subjective characteristics of work have a significant impact on the overall mental health of the nurses and to what extent demographic and occupational factors influence such relationships. This study is an attempt to fill the gap in the research confirming that improving the organization and working climate of nurses can be a strategic goal of hospital management (29).

MATERIALS AND METHODS

The cross-sectional study was conducted in the first 2 weeks of March 2020, in Białystok, in Poland. It included registered nurses working in hospitals and clinics in the Podlaskie Voivodeship. Participation in the study was voluntary, and all procedures were approved by the Bioethical Committee of the Medical University of Bialystok [ref. no APK.002110.2020].

Study Group Selection

The selection of respondents to the study group was based on the register of associated nurses in the District Chamber of Nurses and Midwives in Bialystok. The total number of registered nurses was 6,085 persons (5,990 women and 95 men). The selection criterion was employment based on employment contract in a hospital. Nurses working part-time and on other than employment contract were ruled out.

Study Procedure

The applied study was conducted using paper-based questionnaires. The questionnaires were distributed by researchers during trainings organized by the District Chamber of Nurses and Midwives in Bialystok. Participation was voluntary. Before the study, each nurse was informed about the anonymity of the conducted research, and about the possibility of withdrawing from the study without stating a reason. They were asked to fill out the surveys in their free time within 2 weeks and to send the completed questionnaires in a sealed envelope to the investigators' address. There were 800 questionnaire surveys distributed, resulting in 558 correctly completed questionnaires obtained. The response rate was 69%. There are no known reasons why 242 respondents did not participate in the study. All the demographic data was obtained from surveys in the form of respondents' self-reports. No incentives were used to encourage participation in the study.

Description of the Questionnaire and the Applied Measures

The research tool for health condition assessment was the Goldberg General Health Questionnaire GHQ-28, in the Polish adaptation by Makowska and Merecz (30). The GHQ-28 questionnaire is used to assess the mental health of adults. It allows for the identification of people whose mental condition was subject to temporary or long-term breakdown as a result of experiencing difficulties, problems or as a result of mental illness, and those who are at significant risk of mental health disorders. The GHQ-28 questionnaire, in addition to the overall score, has four measures: somatic symptoms; anxiety/insomnia; social dysfunction and severe depression symptoms. The severity of these negative mental conditions is measured by summing up the answers to specific questions, coded in a dichotomous system. Having considered the foregoing, total measures for individual domains can take values 0–7 points, and 0–28 points for total measures. The higher the GHQ value, the worse the mental health. These measures are standardized by the authors of the questionnaire. The original GHQ-28 and Polish version both have been extensively validated and both have clear scoring guidelines.

The Subjective Work Evaluation Questionnaire (SWEQ) by Dudek et al. was used for assessment of subjective work characteristics (1). This questionnaire is used to measure the subjective perception of work and is designed to measure employees' individual sense of professional stress. It consists of 50 statements describing different characteristics of work. These are numbered from 1 to 5 so as to indicate the extent to which a particular characteristic is onerous (1 - the characteristic is not present in the job, 5 - the highest degree of nuisance). The questionnaire has been extensively validated and has clear scoring guidelines.

Statistical Methods

In the descriptive part, we have prepared the characteristics of the study population in the form of tables containing values of selected descriptive statistics for numerical characteristics or percentage distribution of selected characteristics.

The analysis of the relationship between two numerical (ordinal) characteristics was carried out by determining Spearman's coefficient of rank correlation (r_S) and supplemented

TABLE 1 | Basic demographic characteristic.

Sex[a]	Female	516	92.5%
	Male	42	7.5%
Education[a]	No master's degree	332	59.5%
	Master's degree	226	40.5%
Age (years)[b]		37.3 ± 11.5	22–60
Work experience (years)[b]		11.8 ± 8.8	0–34

[a] *Counts and percent.*
[b] *Mean ± std. dev. & minimum-maximum range.*

TABLE 2 | Mental health of the nurses.

GHQ-28	Mean	Median	Std. dev.	Min	Max
Somatic symptoms	2.26	2	2.28	0	7
Anxiety/insomnia	2.15	1	2.27	0	7
Social dysfunction	1.41	0	2.00	0	7
Severe depression	0.41	0	1.11	0	7
Total	6.23	5	6.35	0	28

by the results of the significance test of the correlation coefficient (p).

We constructed six regression models in which the overall mental health measure was the dependent variable, and the set of independent variables consisted of SWEQ psychometric measures and selected demographic and professional factors of age, education and ward type. Using a progressive stepwise procedure, we then selected optimal model forms in which we took only statistically significant factors into account and interpreted them.

RESULTS

Study Group

The research group consisted of 558 nurses. The vast majority of the respondents were women (92.5%). Nurses younger than 34 years constituted 44.8%, and those older than 51 years – 15.4% of the respondents. Over 40% of the respondents had a master's degree in nursing. Those with work experience shorter than 6 years constituted 35.5% and longer than 17 – 29.6% of all the respondents. The demographic characteristics of the analyzed group of nurses are presented in **Table 1**.

Mental Health of the Nurses

Table 2 presents statistics on the distribution of GHQ-28 measures in the entire study population. As can be seen, mental discomfort is manifested primarily by the presence of somatic symptoms (mean 2.26 points) and anxiety/insomnia (mean 2.15 points), with social dysfunction to a lesser extent, and severe depression being the least manifesting. It should be noted that there is a very significant asymmetry in the distribution of GHQ measures (apart from the overall measure) – the mean values are clearly higher than the medians. In case of social dysfunction and severe depression medians are 0 points, meaning that at least half of the nurses do not experience any mental problems in these areas.

TABLE 3 | Subjective evaluation of negative work features.

Subjective Work Evaluation Questionnaire	Mean	Median	Std. dev.	Min	Max
General stress	129.7	128	35.5	55	233
Work overload	20.8	20	7.1	9	43
Lack of rewards	18.7	19	6.3	8	37
Uncertainty in workplace	17.8	18	5.7	7	35
Social relations	11.3	10	3.5	5	25
Threat	13.3	13	3.7	5	25
Physical burdens	9.8	8	5.3	4	20
Unpleasant work conditions	5.9	3	3.6	3	15
Lack of control	9.4	9	2.9	4	20
Lack of support	5.8	5	2.6	3	14
Responsibility	10.3	10	3.1	4	19

TABLE 4 | Number and percentage of nurses with high levels of negative work characteristics.

Nurses with high level of stress[a]	*N*	%
General stress (>101 pts)	430	77.1%
Threat (>9 pts)	466	83.5%
Responsibility (>7 pts)	442	79.2%
Social relations (>8 pts)	441	79.0%
Lack of rewards (>13 pts)	423	75.8%
Lack of control (>7 pts)	386	69.2%
Uncertainty in workplace (>14 pts)	382	68.5%
Work overload (>16 pts)	371	66.5%
Physical burdens (>7 pts)	363	65.1%
Lack of support (>4 pts)	349	62.5%
Unpleasant work conditions (>4 pts)	253	45.3%

[a] *Thresholds of high level for each SWEQ measure are shown in parentheses.*

Subjective Evaluation of Negative Work Features

The subjective evaluation of negative features of nurses' work was carried out using the 50-position SWEQ questionnaire, based on which the score-based measures of workload in 10 selected aspects are calculated (1). These measures are pejorative in nature – their higher values mean worse result of work evaluation. **Table 3** presents information on the distribution of numerical measures of work features.

The constituent measures in the SWEQ questionnaire were not standardized by the questionnaire authors, thus making direct comparison impossible. Because of that, we have distinguished people who were classified as having a high level of negative work characteristics according to the thresholds established by the authors of the SWEQ questionnaire (1). **Table 4** presents a summary of the numbers and percentages of people with a high level of negative assessments related to a given aspect of work. The results, apart from general stress, were ranked

TABLE 5 | Spearman correlation coefficients between the assessment of negative work characteristics and mental health measures.

Subjective Work Evaluation Questionnaire	GHQ-28				
	Somatic symptoms	Anxiety/insomnia	Social dysfunction	Severe depression	Total
General stress	0.29***	0.37***	0.37***	0.30***	0.39***
Work overload	0.29***	0.37***	0.37***	0.28***	0.39***
Lack of rewards	0.27***	0.31***	0.33***	0.25***	0.35***
Uncertainty in workplace	0.26***	0.31***	0.31***	0.26***	0.34***
Social relations	0.22***	0.27***	0.29***	0.28***	0.30***
Threat	0.19***	0.27***	0.29***	0.22***	0.28***
Physical burdens	0.18***	0.24***	0.22***	0.17***	0.24***
Unpleasant work conditions	0.12**	0.14**	0.12**	0.12**	0.15***
Lack of control	0.25***	0.33***	0.30***	0.25***	0.34***
Lack of support	0.22***	0.26***	0.29***	0.26***	0.30***
Responsibility	0.16***	0.20***	0.22***	0.24***	0.23***

*Statistical significant dependencies: **$p < 0.01$; ***$p < 0.001$.*

from the characteristics most often regarded as negative, to those rarely indicated by the respondents.

Correlations Between the Assessment of Negative Work Features and Mental Health Measures

We then assessed the relationship between work stress measures and mental health measures by determining Spearman's rank correlation coefficients between GHQ-28 and SWEQ measures. The analysis covered the entire population.

Based on the correlation matrix presented in **Table 5**, it can be concluded that the relatively strongest correlations are between the total evaluation of stress at work and the total evaluation of mental health, as well as between *work overload* and total health evaluation ($r_S = 0.39$). Clearly the weakest correlations were found between *unpleasant work conditions* and total health measures ($r_S = 0.12$–0.15).

The Effect of the Overall Measure of Work-Related Stress on Total Mental Health Measure, Including Occupational and Demographic Factors

Based on the correlation analysis presented in **Table 5**, we found that the general measure of stress SWEQ and total mental health measure GHQ-28 are statistically significantly related. However, in order to check if this is not an apparent dependence and to discover what factors influence the strength of this dependence, we conducted a regression analysis in which the dependent variable was the total measure of GHQ-28 and the independent variables were:

- SWEQ general measure of stress at work;
- age – in a dichotomous division into two groups (under the age of 40 and 40 or older);
- education (divided into secondary/bachelor degree vs. higher education);
- ward (Emergency, Internal and Surgical).

TABLE 6 | The effect of the overall measure of work-related stress and education levels on overall mental health.

Predictors	GHQ-28 (total) $R^2 = 18.7\%$ $F = 13.5$ $p = 0.0000$***		
	B (95% c.i.)	*p*	*ß*
General stress at work (SWEQ)	0.073 (0.059; 0.087)	0.0000***	0.41
Education (higher vs. secondary) × general stress at work (SWEQ)	−0.006 (−0.009; −0.002)	0.0032**	−0.11

*R^2, coefficient of determination; F, test statistic and p-value for significance of whole model; B, regression coefficient with 95% c.i., p-value for significance of each regression coefficient; ß, standardize regression coefficient. **$p < 0.01$; ***$p < 0.001$.*

We selected age, education and ward type as occupational and demographic factors, because we found no statistically significant correlation between sex, place of residence and marital status of the respondents and any of the SWEQ and GHQ-28 questionnaire components.

In the first stage, we introduced all independent variables into the model, additionally taking into account the second degree interactions between them. There were a total of four main factors and six interactions in the model. Most of the factors in this model were statistically insignificant, so we decided to search for the optimal form of the model by means of a progressive stepwise regression procedure. The result is a fairly simple model with one main factor and one interaction. It turned out that age and ward do not affect total GHQ-28 measure statistically significantly, neither as separate factors nor in interactions. The model presented in **Table 6** includes two factors: SWEQ general stress at work and education, but in interaction with the SWEQ general stress measure.

The model explains a total of 18.7% variation of GHQ-28 in the study population. Detailed interpretation of model parameters is as follows:

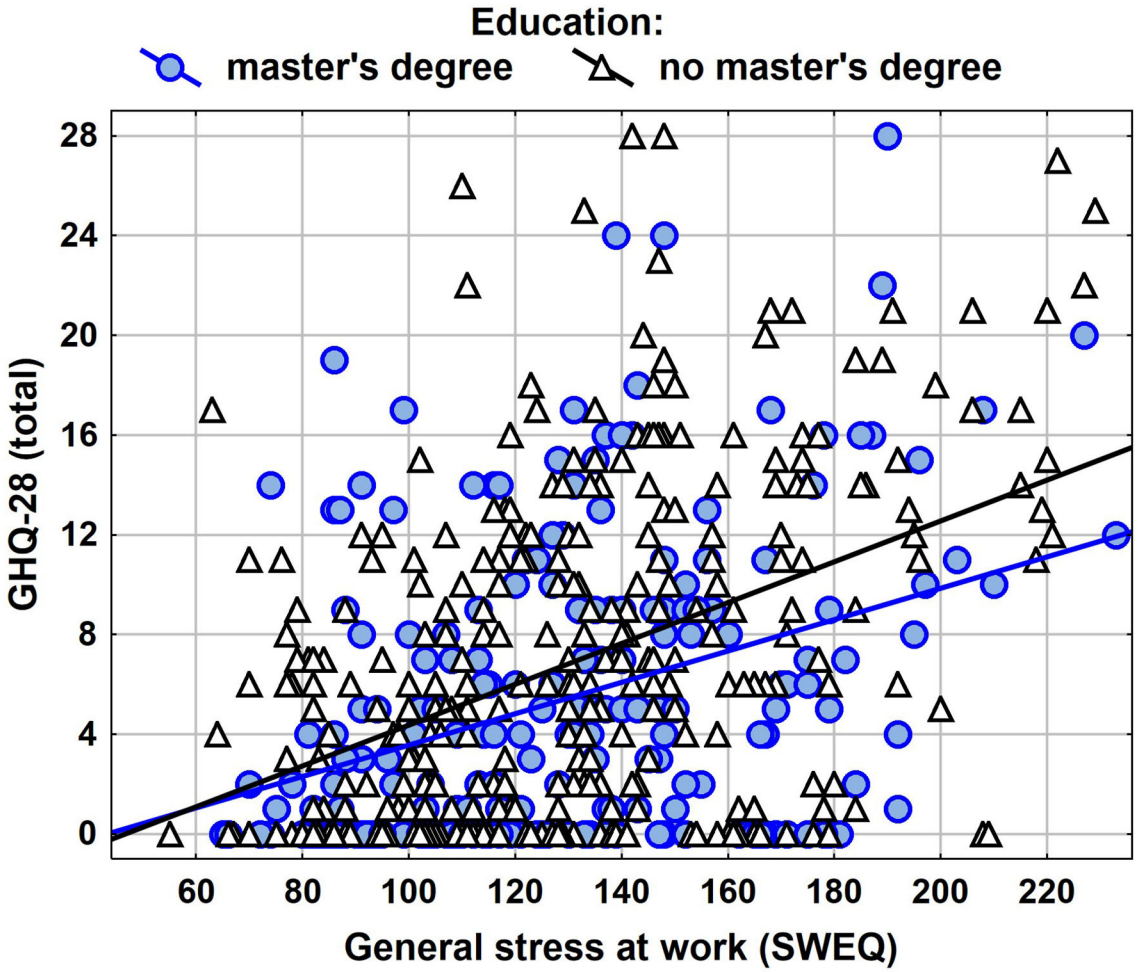

FIGURE 1 | NImpact of SWEQ general measure in interaction with education level on GHQ-28 total.

- As the general measure of stress at work increases by 1 point, the total GHQ-28 increases, on average by about 0.073 point.
- The effect of the SWEQ general stress measure on the total GHQ-28 is education-dependent.

Due to relatively simple regression model form, it is possible to illustrate the results in the form of a scatter plot (**Figure 1**). This shows both the effect of the SWEQ general measure and its correction for education level. Looking at the position of the simple regressions, we can see a stronger influence of the SWEQ general measure on the GHQ-28 total measure among nurses without higher education.

The Effect of Partial Psychometric Measures on the Overall Mental Health Level

In the next analysis, we used regression analysis to assess which of the ten partial psychometric measures determined using the SWEQ questionnaire have a statistically significant effect on the overall GHQ-28 measure and which percentage of GHQ-28 variability can be explained when synthesizing information about the level of stress experienced at work.

The preliminary analysis of the correlation coefficients in **Table 5** shows that all SWEQ components are statistically significantly related to the GHQ-28 total measure. Subsequently, we constructed a regression analysis model in which we adopted the total GHQ-28 as a dependent variable, and we introduced all 10 SWEQ partial measures as independent variables.

The full model, with all the SWEQ measures allowed explaining the variability of GHQ-28 mental health status to 19.2%. However, only two components: *work overload* and *responsibility*, were statistically significant ($p < 0.05$). A model in which as many as eight independent characteristics were statistically insignificant could not be the subject of final conclusions. Because of this, by applying a progressive stepwise regression procedure, we selected five SWEQ measures that were the most important determinants of the nurses' mental state: *work overload*, *lack of rewards*, *social relations*, *lack of support* and *responsibility*. **Table 7** shows the model that was subject to final interpretation.

TABLE 7 | The effect of partial psychometric measures SWEQ on the overall mental health level.

Predictors	GHQ-28 (total) $R^2 = 19.2\%$ $F = 25.9$ $p = 0.0000$***		
	B (95% c.i.)	*p*	*ß*
Work overload	0.202 (0.093; 0.312)	0.0003***	0.23
Lack of rewards	0.125 (0.005; 0.245)	0.0419*	0.12
Social relations	0.259 (0.052; 0.465)	0.0142*	0.14
Lack of support	0.267 (0.003; 0.530)	0.0472*	0.11
Responsibility	−0.241 (−0.464; −0.017)	0.0348*	−0.12

*R^2, coefficient of determination; F, test statistic and p-value for significance of whole model; B, regression coefficient with 95% c.i., p-value for significance of each regression coefficient; ß, standardize regression coefficient. *p < 0.05; ***p < 0.001.*

TABLE 8 | The effect of partial psychometric measures SWEQ and level of education on the overall mental health level.

Predictors	GHQ-28 (total) $R^2 = 20.7\%$ $F = 35.4$ $p = 0.0000$***		
	B (95% c.i.)	*p*	*ß*
Work overload	0.196 (0.104; 0.288)	0.0000***	0.22
Social relations	0.254 (0.060; 0.449)	0.0106*	0.14
Lack of support	0.291 (0.036; 0.547)	0.0252*	0.12
Education (higher vs. secondary) × unpleasant work condition	−0.149 (−0.220; −0.078)	0.0000***	−0.16

*R^2, coefficient of determination, F, test statistic and p-value for significance of whole model; B, regression coefficient with 95% c.i., p-value for significance of each regression coefficient; ß, standardize regression coefficient. *p < 0.05; ***p < 0.001.*

Taking into account the standardized regression coefficient values *ß*, we found that the most important factor is *work overload*, while the importance of the others is similar. This model explains more than 19.2% of the variability of the GHQ-28 total measure.

The Influence of Partial Psychometric Measures on the Overall Level of Mental Health, Including Occupational and Demographic Factors

The model developed in this section is an extension of the results presented in the model presented in **Table 7**, by introducing demographic and occupational variables as additional control factors. We introduced three factors (ward, education, age) and thirty interactions between each of these factors and the SWEQ partial measures. Then, using a progressive stepwise regression procedure, we determined statistically significant factors. The results are presented in **Table 8**.

As can be seen, the first three measures have values not too different from those in the model showed in **Table 7**. Absence of *lack of reward* and *responsibility* measure effect, and the emergence of a statistically significant interaction between education and the effect of *unpleasant work conditions* on the total measure of mental health are significant differences. A negative coefficient for the interaction effect means that the effect of the unpleasant working conditions factor on the total GHQ-28 measure is weaker among nurses with higher education and stronger among nurses with secondary education.

DISCUSSION

The aim of our study was to determine the effect of stress experienced by nurses on their mental health in interaction with occupational and demographic factors. Generally speaking, nurses consider their work to be highly stressful. This is reflected by the high position of nursing in rankings of the most stressful professions. According to studies conducted in Poland, nursing is in the second group of the most stressful occupations (3), and in the USA, nursing is in the top five most stressful occupations according to the scale developed by the Occupational Information Network (31).

In our survey, as many as 77.1% of all respondents described the perceived overall level of stress caused by work as very high. Some of the components of the SWEQ questionnaire were assessed as very high by an even higher percentage of respondents. *Threat* results in very high stress in 83.5% of all respondents, with *responsibility* and *social relations* inducing this ranked at 79.0 and 79.2%, respectively. Only *unpleasant work conditions* clearly differs from the other measures, as this was indicated as a highly stressful factor by 45.3% of all respondents. The fact that the percentage of results considered high is very high is due to the fact that the questionnaire authors set standards while taking different professions into account (1). This means that nurses see relatively many negative characteristics in their work compared to other professions. In similar studies carried out in hospitals in other parts of the country, the most common stressors were *responsibility, work overload* and *threat* (23, 32). Most of the studies conducted among nurses in different countries indicated *work overload* as the most stressful characteristic of work. In the case of the incidence of the remaining stressful characteristics in the nurses' work, no significant regularity was observed among the published study results (33–35). This is probably due to cultural differences and the use of different research tools, as there are no popular international questionnaires such as the GHQ series for subjective evaluation of work characteristics.

In general, nurses are quite satisfied with their mental health. At least ¼ of the respondents do not complain about any mental discomfort, and at least half of them do not show social dysfunction. As many as ¾ of all respondents do not experience the symptoms of depression. However, the distribution of GHQ-28 mental health measures in our studies is characterized by high asymmetry. This was clearly notable in the case of somatic symptoms and anxiety/insomnia where ¼ of the respondents assess their condition significantly worse than average. By selecting GHQ-28 score of 6 as the cut-off point, it can be concluded that 39.9% of all nurses suffer from mental disorders. Somatic symptoms were the most frequent and severe depression was the least frequent. In this respect, our results differ from

the results obtained in the study conducted in Iran (36), where 45.4% of all nurses complained about mental disorders, but social dysfunctions were by far the most common disorder, and somatic symptoms were only ranked third. In studies carried out among Greek nurses, depressive conditions were much more frequent than in our country, whereas anxiety/insomnia were at similar level (37). In Lithuania, Poland's neighbor, as much as 60.4% of the surveyed nurses assessed their mental health as poor (38). Very similar results to ours were obtained in a study conducted in Poland in the neighboring Lublin Voivodeship, where 38.1% of all nurses suffered from mental disorders, and while severe depression was the least frequent, the remaining components were at a similar level (39).

Our work indicated that negative characteristics of the work are associated with the assessment of bad mood. The correlation matrix presented in **Table 5** shows the existence of statistically significant correlations between all negative work characteristics (SWEQ) and measures of mental health (GHQ-28). All correlations are positive and highly statistically significant, with the exception of *unpleasant working conditions* and GHQ-28 questionnaire partial measures, which are only statistically significant. Similar relationships were obtained in studies conducted by other researchers (40, 41). Furthermore, very similar results were obtained in studies conducted in Japan (42). Indeed, even the strength of most correlations is almost identical.

The co-occurrence of negative work characteristics and poor well-being does not indicate how negative work characteristics can affect the mental health of nurses. Using regression models, we studied how the subjective overall measure of stress at work, demographic and occupational factors, separately and in interaction with each other, can influence the GHQ-28 total measure of nurses' mental health. We did not find any effect of age or ward type, alone or in interaction with the nurses' assessment of their mental condition. The final version of the model showed that only the SWEQ general measure of stress alone and in interaction with education affects the overall mental well-being of the GHQ-28 surveyed nurses. While the increase in the overall SWEQ stress at work measure results in worse overall psychological well-being of the subjects, after analyzing interactions with education, it can be concluded that nurses with a higher education level demonstrate better mental capacity to withstand workplace stress. Similar results were obtained in studies carried out in Silesia (23). However, while studies carried out in other countries confirm the negative effect of occupational stress on the mental health of nurses (43), the effect and interaction of other factors is different. For example, some researchers show interaction with the type of ward or seniority (9, 21, 44, 45).

Examination of the effect of the partial psychometric measures from the SWEQ questionnaire on the overall GHQ-28 measure showed that the most significant effect was demonstrated by *work overload*. *Lack of rewards, social relations, lack of support* and *responsibility* showed lower effect, and the other measures did not affect the GHQ-28 overall measure. The increase in stress caused by *work overload, lack of rewards, social relations* and *lack of support* caused deterioration in the mental well-being of nurses, whereas in the case of the *responsibility* measure, the coefficient was negative. This means that an increase in the level of stress induced by the sense of responsibility improves the psychological well-being of the studied nurses. This is an apparent paradox, since as many as 79.2% of all respondents described *responsibility* as a highly stressful factor, and it was the second most stressful factor in terms of frequency. After deeper analysis, we have come to the conclusion that an increase in the sense of responsibility alone, with no changes to the other SWEQ measures, can actually mean greater certainty for the nurse at work and have a positive effect on overall mental health. *Work overload, lack of rewards, social relations, lack of support* negative effect and the positive impact of increased sense of *responsibility* on the mental health of nurses have been presented in other studies (46–48). These results are in line with the theoretical Job Demand Control Support model (18) and Effort Imbalance Model (19).

The final part of the analysis was to examine the influence of partial psychometric measures in interaction with occupational and demographic factors on the overall GHQ-28 measurement. The constructed regression model again showed the strong effect of *work overload*, with the much weaker effect of *social relations* and *lack of support* on the deterioration of nurses' well-being, as measured by the overall measure in the GHQ-28 questionnaire. However, the most interesting result was the significant effect of education in interaction with *unpleasant work condition*. The negative coefficient for this interaction can be interpreted in such a way that nurses with higher education are more resistant to the negative effect of *unpleasant work* conditions on overall mental health self-assessment. The effect of higher education as a factor increasing the nurse resistance to stress at work and its positive effect on well-being self-assessment was confirmed by other researchers (49, 50). The special interaction between education and *unpleasant work conditions* resulting from our research deserves attention and can be a complement to early research.

Summarizing the above, the stress in the workplace determined by the general measure of the Subjective Work Evaluation Questionnaire has a negative effect on the self-assessment of the mental health of the nurses by means of the GHQ-28 questionnaire. Among the partial measures of the SWEQ questionnaire, *work overload* had strong effect, whereas the *lack of rewards, social relations* and *lack of support* had a weak negative effect on the overall mental health self-assessment of nurses. *Responsibility* was an exception that had a positive effect on the nurses' well-being. Among occupational and demographic factors, only higher education in relation to secondary education in interactions with the general measure of stress and *unpleasant work conditions* had a positive effect on the overall mental health self-assessment of nurses, determined using the GHQ-28 questionnaire.

CONCLUSIONS

1. Stress caused by work overload is the factor most negatively influencing the self-assessment of mental health in nurses. A greater sense of responsibility for one's work has a positive effect on the self-assessment of the mental health in nurses.

2. Higher education in relation to secondary education is a factor that positively affects the self-assessment of mental health in nurses. Nurses with higher education show better tolerance concerning unpleasant work conditions as a stress factor negatively affecting their mental health.
3. The results of our study provide a clear message to the hospital management, consistent with other studies (29). Improving the organization and atmosphere at work of nurses toward reducing perceived work overload and increasing the responsibility of nurses can have a positive impact on the mental health of nurses. Encouraging nurses to improve their education can result not only in an obvious improvement in staff qualifications, but also in better resistance to stressors in the workplace and, consequently, in better staff well-being. Both measures can have a positive impact on the quality of care provided by nurses and can reduce staff turnover.

METHODOLOGICAL LIMITATIONS

The sample used, study design (cross-sectional study), self-reported style questionnaires and are significant limitations of the study. The research was conducted only in a single region of Poland. The respondents were completing questionnaires remotely, which could lead to data bias and low response rate. The reasons for 31% of all invited persons not taking part in the study are unknown due to the manner the study was conducted.

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 Bioethical Commitee of Medical University of Bialystok, Poland. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

KK: concept of the research, design of article structure, conducting of the research, review of the literature, results analysis, and writing the article. EK-K: review of the literature and review of article drafts. MS: statistical analysis. All authors contributed to the article and approved the submitted version.

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Serum Levels of FGF21, β-Klotho, and BDNF in Stable Coronary Artery Disease Patients With Depressive Symptoms: A Cross-Sectional Single-Center Study

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Wu Y, Chen Z, Duan J, Huang K, Zhu B, Yang L and Zheng L (2021) Serum Levels of FGF21, β-Klotho, and BDNF in Stable Coronary Artery Disease Patients With Depressive Symptoms: A Cross-Sectional Single-Center Study. Front. Psychiatry 11:587492. doi: 10.3389/fpsyt.2020.587492

Background: The incidence of depressive symptoms (DS) in patients with stable coronary artery disease (SCAD) is significantly higher than those in healthy population, and that DS are independent risk factors for cardiovascular events. Previous studies have reported that fibroblast growth factor 21 (FGF21), β-klotho, mature brain-derived neurotrophic factor (mBDNF), and BDNF precursor (proBDNF) play important roles in the pathogenesis and treatment of coronary heart disease and depression. With this in mind, the present study aimed to clarify the relationship between FGF21, β-klotho, mBDNF, and proBDNF and SCAD with comorbid depression, in addition to also exploring the underlying mechanisms of these disease processes.

Methods: A total of 116 patients with SCAD and 45 healthy controls were recruited. Patients with SCAD were further divided into two subgroups based on the Zung Self-Rating Depression Scale (SDS), which were characterized as those with no DS (NDS) and those with DS. Baseline data were collected, and serum levels of FGF21, β-klotho, mBDNF, and proBDNF were determined.

Results: In SCAD patients, Gensini scores—denoting the degree of coronary arteriostenosis—were significantly greater in the DS group than in the NDS group. There was also a positive correlation between the Gensini scores and the SDS scores. Patients in the SCAD group demonstrated a lower serum FGF21. Serum β-klotho, mBDNF, and mBDNF/proBDNF were also significantly lower in the DS group than in the NDS group. Furthermore, β-klotho and mBDNF were negatively correlated with the SDS scores. Additionally, SCAD patients were divided into lower- and higher-level groups using hierarchical cluster analysis, with the results highlighting that patients in the lower mBDNF group had a higher incidence of DS.

Conclusions: The depression score was positively correlated with the severity of coronary artery stenosis, and serum FGF21, β-klotho, mBDNF, and proBDNF were closely related to the development of DS in patients with SCAD. These observations suggest FGF21, β-klotho, mBDNF, and proBDNF as potential diagnostic and/or therapeutic targets for SCAD with co-morbid depression.

Keywords: stable coronary artery disease, depressive symptoms, fibroblast growth factor 21, β-Klotho, brain-derived neurotrophic factor

INTRODUCTION

Depression is a major cause of disability worldwide and has a huge impact on other chronic diseases (1). An epidemic survey demonstrated that approximately 34.6–51% of Chinese patients with coronary heart disease (CHD) also suffered from depression (2). Of these depression cases, major depressive disorder accounted for 3.1–11.2% (2). To put this into context, the incidence of depression in the general population is only 3.2% (3). The Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5) is the gold standard for the diagnosis of clinical depression (4). Furthermore, the depressive symptoms (DS) could be assessed by self-reported clinical scale (4). Currently, approximately 20% of patients that underwent coronary angiography following chest pain demonstrated normal or near-normal coronary arteries, and the chest pain that they had experienced could not be explained by other organic diseases. A study by Christoph et al. enrolled 253 patients to evaluate anxiety, depression, hypochondria, and somatoform disorders using well-validated questionnaires. The results suggested that patients with non-cardiac chest pain were more likely to develop psychopathological symptoms than healthy individuals (5). Stable coronary artery disease (SCAD) is the most common type of ischemic heart disease (6). Although the prognosis for people suffering from SCAD is good, quality of life and health conditions decline drastically in patients suffering from SCAD with co-morbid depression (7, 8). Depression is also one of the independent risk factors for adverse cardiovascular events (7–9).

We previously reported that the incidence of moderate/severe DS in patients with SCAD was 18.8% (10). Further analyses demonstrated that elderly patients were much more likely to experience DS (10). It has been proposed that abnormalities in low-density lipoprotein (LDL), high-density lipoprotein (HDL), and creatinine (Cr) could contribute to DS (10). Interestingly, CHD with co-morbid depression is underpinned by a complex multifactorial process that includes inflammation, endothelial dysfunction, platelet activation, and gut microbiota disturbance, which form a complex pathogenic network (11, 12). Due to limited attention in the clinic, DS are often masked by physical illness. They can also manifest as severe somatic symptoms that are inconsistent with disease severity. For these reasons, diagnosis and appropriate intervention are problematic for clinicians. Therefore, it is of great importance to identify novel, effective biomarkers for the diagnosis and treatment of SCAD with comorbid depression.

Recent studies have indicated that fibroblast growth factor 21 (FGF21) plays an important role in the processes of CHD and depression (13–16). The FGF21 protein is a new member of the FGF protein family and is mainly derived from the liver, kidneys, adipocytes, and cardiomyocytes. The FGF21 protein functions both in the endocrine system and as a cytokine, and it can be released into the circulation to exert its biological effects through specific binding to the co-receptor, β-klotho (17–19). The expression of FGF21 is significantly influenced by β-klotho, which exhibits tissue-specific expression in the liver, heart, and nervous system (20, 21). In addition to its role as a cytokine, FGF21 has an important role in regulating lipid metabolism and inflammation, thereby preserving endothelial function and delaying the development of cardiovascular disease (13–15). Furthermore, Liu and coworkers demonstrated a significant negative correlation between the level of FGF21 in cerebrospinal fluid and depression in male subjects, suggesting that FGF21 has beneficial effects on neuroprotection and emotional regulation (16). Collectively, the above studies indicate that FGF21 is involved in the development of CHD and depression.

Brain-derived neurotrophic factor (BDNF) is an important member of the neurotrophic factor family, which regulates neuronal development and plasticity. BDNF is processed by the Golgi complex from the N-terminal glycosylation precursor protein, BDNF precursor (proBDNF), and released into the extracellular environment (22). Mature BDNF (mBDNF) is a neuroprotective factor that has been associated with neuronal survival, plasticity, and differentiation. Diminished expression of neurotrophic factors represented by mBDNF and the associated impairment of neuroplasticity may directly exacerbate depression (22–25). Interestingly, proBDNF exerts biological effects that are distinct from mBDNF. Specifically, proBDNF upregulates p53 expression and initiates apoptosis through its involvement in the p75 neurotrophin receptor (p75NTR)-activated c-Jun amino-terminal kinase (JNK) pathway, thereby interfering with neurotransmitter release and inhibiting axonal outgrowth (26, 27). Furthermore, mBDNF, as a novel pro-angiogenic factor in CHD, has attracted attention as a contributor to the growth of vascular endothelial cells and proliferation of ischemic endothelial cells. Thus, it appears that mBDNF plays an important role in atherosclerosis and ischemic cardiomyopathy, amongst other diseases (28–30).

In this study, we measured the serum levels of FGF21, β-klotho, mBDNF, and proBDNF in SCAD patients with or without DS and compared this to results from 45 healthy controls (HCs). These findings were used to investigate the relationship between

these factors and SCAD with co-morbid depression and to explore the underlying developmental mechanisms.

MATERIALS AND METHODS

Patients and Study Design

A cross-sectional single-center study was conducted using patients from the Third Affiliated Hospital of Soochow University. From July 2017 to October 2018, a total of 116 patients with SCAD were recruited. Forty-five HC subjects were also recruited from the medical examination center. The study was approved by the Ethics Committee of the Third Affiliated Hospital of Soochow University (No. 2017015) and was registered in the Chinese Clinical Trial Registry (ChiCTR1900020594). This study incorporated secondary analyses of clinical trial data from our previous study and used the same registration number (10).

Patients with SCAD were enrolled if they conformed to at least one of the following criteria: (1) clinically diagnosed with myocardial infarction (>3 months); (2) demonstrated at least one coronary artery stenosed by >50% by coronary angiography; (3) demonstrated coronary artery stenosis or myocardial infarction after chest pain; or (4) had undergone coronary artery bypass graft or percutaneous coronary intervention (>3 months).

Patients were excluded from the study if they had experienced: (1) a history of depression or other psychiatric disorders, and on anti-depressant or psychotropic medication; (2) acute myocardial infarction during hospitalization (manifested by electrocardiographic changes and/or elevated myocardial enzymes); (3) myocardial infarction or cardiac surgery in the past 3 months; (4) an acute infectious disease in the month prior to enrollment; (5) other severe cardiovascular diseases (e.g., acute pericarditis, myocarditis, end-stage heart failure, and secondary heart disease); (6) diseases seriously affecting life expectancy (e.g., connective tissue disease, cancer, drug abuse, and dementia); (7) pregnancy; (8) recent major stressful life events; or (9) an inability to complete the depression scale assessment or blood sampling.

Physical and Clinical Examination

Baseline data were obtained by carrying out interviews, accessing medical records, and assessing age, sex, body mass index (BMI), blood pressure, diabetes history, smoking history, and β-blocker and statin use. Fasting venous blood samples were collected and sent to our laboratory. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum total bilirubin (STB), HDL, LDL, albumin/globulins (A/G), blood urea nitrogen (BUN), Cr, and hemoglobin (Hb) were measured. White blood cell (WBC) counts, neutrophil percentage (N%), and lymphocyte percentage (L%) were also assessed. All patients underwent echocardiography to obtain left ventricular ejection fraction (LVEF). All tests were performed and reported by the same physician in the hospital. In addition, all patients underwent coronary angiography via the brachial or radial artery, and the results were interpreted by two experienced cardiologists. The degree of luminal stenosis in the left main, left anterior descending, circumflex, and right coronary arteries were recorded, and the Gensini score was calculated to quantitatively evaluate the degree of coronary artery stenosis.

Assessment of DS

Patients with SCAD were evaluated for DS using the Zung Self-Rating Depression Scale (SDS) during hospitalization. SDS is one of the most widely used self-reported clinical scale and its validity have been established in clinical depression evaluation (31–33). It has good internal consistency and validity, encompassing most DSM-IV criteria for major depression (32). Consisting of 20 items, the SDS is scored on a four-point scale to assess the psychological and physical symptoms of depression. A standard score is obtained by multiplying the total score by 1.25. Patients with SCAD were further categorized into two subgroups based on their standard scores. These subgroups included patients with no DS (NDS) (score $\leq$ 52) and those with DS (score $\geq$ 53).

Enzyme-Linked Immunosorbent Assay (ELISA)

A volume of 10 ml morning fasting venous blood was collected from the cephalic vein and placed in a non-anticoagulated biochemical test tube. Blood samples were centrifuged at 3,000 r/min for 4 min to obtain serum. A volume of 110 μl of serum was added to each tube and stored at −80°C until measurements were carried out.

Serum levels of FGF21 (Camilo, H-KMLJ31425, the detection range, recovery rate, intra and inter-assay coefficients of variation were 3.75–2,000 pg/ml, 70–110, ≤15, and ≤15%, respectively), β-klotho (Camilo, H-KMLJ39385, the detection range, recovery rate, intra and inter-assay coefficients of variation were 1.56–20 ng/ml, 70–110, ≤15, and ≤15%, respectively), mBDNF (Camilo, H-KMLJ39649, the detection range, recovery rate, intra and inter-assay coefficients of variation were 0.78–50 ng/ml, 70–110, ≤15, and ≤15%, respectively), and proBDNF (Camilo, H-KMLJ31139, the detection range, recovery rate, intra and inter-assay coefficients of variation were 0.312–30 ng/ml, 70–110, ≤15, and ≤15%, respectively) were determined by ELISA. The operation steps were carried out according to the manufacturer's instructions: (1) aluminum slats were removed from the foil bag after 20 min at room temperature; (2) standard wells, sample wells, and blank wells were set, and standard wells were loaded with 50 μl of standards; (3) 10 μl of sample and 40 μl *diluent* were added to the sample wells; (4) 50 μl of horseradish peroxidase-labeled detection antibody was added to the standard wells and the sample wells; (5) the plate was sealed and incubated at 37°C in a water bath or thermostat for 60 min; (6) the liquid was discarded and washing solution was added to each well before leaving to stand for 1 min; (7) washing solution was removed; (8) steps (6) and (7) were repeated five times; (9) 50 μl of each of substrates A and B were added to each well and incubated at 37°C for 15 min in the dark; and (10) 50 μl of stop solution was added to each well, and the absorbance was measured immediately at 450 nm. The concentration of each factor was obtained according to the optical density-concentration standard curve.

Statistical Analysis

Statistical analysis was performed using SPSS 24.0 and GraphPad Prism 7.0. Data were expressed as mean ± standard deviation, and each parameter was tested for normality. If the data were normally distributed, the means of the two groups were compared using a *t*-test. If data were not normally distributed, the means of the two groups were compared using the Mann–Whitney *U*-test. Categorical data were expressed as the rate (%), and a chi-squared test was used. A correlation analysis was performed using the Pearson correlation, Spearman correlation and multiple linear regression analysis. A two-sided $p < 0.05$ was considered statistically significant for all tests.

TABLE 1 | Clinical characteristics of HC and SCAD subjects.

Parameters	HC ($n = 45$)	SCAD ($n = 116$)	*P*-value
Age (years)	61.2 ± 8.7	63.4 ± 9.0	0.166
Male (%)	32(71.1)	84(72.4)	0.869
SBP (mmHg)	135 ± 19	135 ± 18	0.936
DBP (mmHg)	78 ± 11	79 ± 11	0.433
BMI (kg/m^2)	23.7 ± 1.7	24.6 ± 3.0	0.022
ALT (U/L)	19.4 ± 7.9	25.1 ± 14.4	0.018
AST (U/L)	22.5 ± 7.0	23.7 ± 7.9	0.344
STB (μm/L)	12.8 ± 4.3	10.2 ± 4.4	0.001
HDL (mmol/L)	1.27 ± 0.29	1.05 ± 0.23	<0.001
LDL (mmol/L)	2.56 ± 0.53	2.04 ± 0.69	<0.001
A/G	1.71 ± 0.22	1.64 ± 0.30	0.054
BUN (mg/dl)	5.79 ± 2.10	4.98 ± 1.86	0.019
Cr (μmmol/L)	79.5 ± 11.3	80.1 ± 44.4	0.901
Hb (g/L)	147.4 ± 13.9	138.6 ± 12.3	<0.001
WBC (×10^9/L)	5.54 ± 1.31	6.51 ± 1.51	<0.001
N (%)	59.9 ± 7.7	63.2 ± 8.4	0.026
L (%)	33.2 ± 7.1	27.6 ± 8.7	<0.001

A/G, albumin/globulins; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; BUN, blood urea nitrogen; Cr, creatinine; DBP, diastolic blood pressure; Hb, hemoglobin; HC, healthy controls; HDL, high density lipoprotein; L%, lymphocyte percentage; LDL, low density lipoprotein; N%, neutrophil percentage; SCAD, stable coronary artery disease; SBP, systolic blood pressure; STB, serum total bilirubin; WBC, white blood cell. After tested for normality, the means of ALT, AST, A/G, and Cr of the two groups were compared using the Mann–Whitney U-test, while others were compared using a t-test and a chi-squared test (for categorical data).

RESULTS

Comparison Between the Clinical Characteristics of HC and SCAD Subjects

There were no significant differences in age, gender, blood pressure, AST, A/G, Cr, and N% between the two groups ($p > 0.05$), while the SCAD group had a greater BMI, a greater ALT and WBC, and lower STB, HDL, LDL, BUN, Hb, and L% levels ($p < 0.05$; **Table 1**).

The concentration of FGF21 in the SCAD group was significantly lower than that observed in the HC group ($p = 0.039$). The serum concentrations of β-klotho, mBDNF, proBDNF, and mBDNF/proBDNF were decreased in the SCAD group. However, no statistically significant difference was identified when compared with the HC group ($p > 0.05$; **Figure 1**).

Comparison of the Clinical Characteristics of SCAD Patients With and Without DS

There were no significant differences in age, gender, blood pressure, BMI, diabetes history, smoking history, ALT, AST, STB, HDL, LDL, A/G, BUN, Cr, Hb, L%, N%, LVEF, or β-blocker and statin use between SCAD patients with and without DS ($p >$

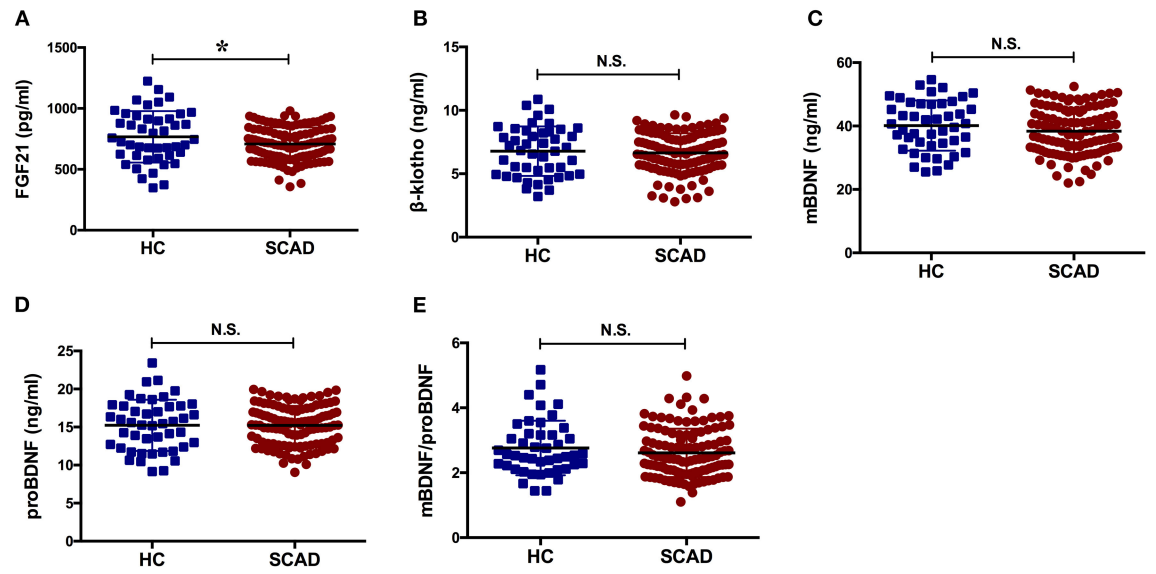

FIGURE 1 | Serum levels of the factors in HC and SCAD. BDNF, brain derived neurotrophic factor; FGF21, fibroblast growth factor 21; HC, healthy controls; N.S., no significance; SCAD, stable coronary artery disease. The means of the two groups were compared using a *t*-test. The concentration of FGF21 in the SCAD group was significantly lower than that observed in the HC group [(707.80 ± 136.40) pg/ml vs. (766.62 ± 211.36) pg/ml, $p = 0.039$].

0.05). The group with DS had lower WBC counts and greater Gensini scores ($p < 0.05$; **Table 2**).

The serum concentration of FGF21 in the group with DS was significantly greater than that observed in the group with NDS ($p = 0.039$), while the serum concentrations of β-klotho, mBDNF, and mBDNF/proBDNF were significantly lower ($p = 0.041$, $p = 0.021$, and $p = 0.029$, respectively). ProBDNF levels were increased in the group with DS, but there was no statistically significant difference when compared with the group with NDS ($p > 0.05$; **Figure 2**).

Correlation Analysis Between Serum Concentrations, Gensini Scores, and the SDS

The Spearman correlation analysis demonstrated a positive correlation between the Gensini scores and the SDS scores, and the correlation coefficient (r) was 0.168 ($p = 0.047$; **Figure 3**). There was no significant correlation between FGF21, β-klotho, mBDNF, proBDNF, and mBDNF/proBDNF concentrations, and the Gensini scores (**Figure 4**). β-klotho and mBDNF levels were negatively correlated with the SDS scores ($r = -0.199$, $p = 0.033$; and $r = -0.206$, $p = 0.027$, respectively; **Figure 5**). The multiple linear regression analysis showed that the serum levels of those factors were not correlated with the Gensini scores ($F = 0.576$, $p = 0.681$), and none of the variables were significant predictors of Gensini score ($p > 0.05$; **Table 3**). The multiple linear regression analysis built a significant model to predict SDS score based on the serum levels of those factors ($F = 2.492$, $p = 0.047$), but the coefficient of determination was low ($R^2 = 0.082$). Significant predictor for a higher SDS score was the lower β-klotho levels serum ($B = -0.772$, $p = 0.042$; **Table 4**).

Comparison of the Incidence of DS in SCAD Patients With Lower or Higher FGF21, β-klotho, mBDNF, or proBDNF Levels

According to the concentration of each of FGF21, β-klotho, mBDNF, proBDNF, and mBDNF/proBDNF, 116 patients with SCAD were further divided into a lower level group and a higher level group using a hierarchical cluster analysis (**Supplementary Figures 1–4**). There were 84 patients in the lower level FGF21 group and 32 patients in the higher level FGF21 group. The threshold for the higher level FGF21 group was 804.05 pg/ml. There were 48 patients in the lower level β-klotho group and 68 patients in the higher level β-klotho group, which had a threshold concentration of 6.37 ng/ml. There were 84 patients in the lower level mBDNF group and 32 patients in the higher level mBDNF group, in which the threshold concentration was 43.61 ng/ml. There were 32 cases in the lower level proBDNF group and 84 cases in the higher level proBDNF group, in which the threshold concentration was 13.15 ng/ml. Importantly, the chi-squared test demonstrated that the patients in the lower level mBDNF group had a higher incidence of DS than the higher level group ($\chi^2 = 5.023$, $p = 0.025$; **Table 5**).

TABLE 2 | Clinical characteristics of SCAD patients with or without depressive symptoms.

Parameters	Without depressive symptoms ($n = 30$)	With depressive symptoms ($n = 86$)	*P*-value
Age (years)	62.0 ± 10.5	63.9 ± 8.5	0.382
Male (%)	22 (64.7)	62 (72.1)	0.896
SBP (mmHg)	134 ± 18	135 ± 19	0.868
DBP (mmHg)	80 ± 12	79 ± 10	0.799
BMI (kg/m^2)	25.0 ± 3.0	24.4 ± 3.0	0.429
Diabetes mellitus (%)	6 (20.0)	32 (37.2)	0.084
Current smoking (%)	10 (33.3)	32 (37.2)	0.704
ALT (U/L)	27.9 ± 17.2	24.1 ± 13.3	0.292
AST (U/L)	25.1 ± 8.7	23.3 ± 7.5	0.287
STB (μm/L)	10.9 ± 3.5	10.0 ± 4.6	0.362
HDL (mmol/L)	1.00 ± 0.21	1.07 ± 0.24	0.120
LDL (mmol/L)	2.12 ± 0.77	2.01 ± 0.66	0.467
A/G	1.72 ± 0.38	1.61 ± 0.25	0.247
BUN (mg/dl)	4.76 ± 1.16	5.06 ± 2.05	0.443
Cr (μmmol/L)	81.4 ± 31.8	79.6 ± 48.2	0.162
Hb (g/L)	138.3 ± 13.3	138.8 ± 12.0	0.859
WBC (×10^9/L)	7.03 ± 1.74	6.33 ± 1.39	0.029
N (%)	61.4 ± 6.9	63.8 ± 8.9	0.183
L (%)	29.1 ± 9.5	27.1 ± 8.4	0.284
Medications (%)			
β blockers	7 (23.3)	36 (41.9)	0.070
Statins	13 (43.3)	41 (47.7)	0.681
LVEF (%)	62.1 ± 2.8	61.5 ± 5.4	0.570
Gensini scores	19.72 ± 8.96	30.71 ± 23.40	<0.001

A/G, albumin/globulins; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; BUN, blood urea nitrogen; Cr, creatinine; DBP, diastolic blood pressure; Hb, hemoglobin; HC, healthy controls; HDL, high density lipoprotein; L%, lymphocyte percentage; LDL, low density lipoprotein; LVEF, left ventricular ejection fraction; N%, neutrophil percentage; SCAD, stable coronary artery disease; SBP, systolic blood pressure; STB, serum total bilirubin; WBC, white blood cell. After tested for normality, the means of ALT, AST, A/G, Cr, LVEF, and Gensini scores of the two groups were compared using the Mann–Whitney U-test, while others were compared using a t-test and a chi-squared test (for categorical data).

DISCUSSION

The results of this study indicate a positive correlation between depression scores and the severity of coronary artery stenosis. Similarly, the serum levels of FGF21, β-klotho, mBDNF, and proBDNF are closely related to the development of DS in patients with SCAD. In addition, β-klotho and mBDNF levels were negatively correlated with the SDS scores, and the incidence of DS was significantly increased in patients with lower serum mBDNF.

It is well-recognized that the incidence of depression in patients with cardiovascular disease is significantly greater than that observed in the healthy population. Psychological disorders, such as depression, can reduce the quality of life for patients and can increase the risk of cardiovascular death (34, 35). These observations reassert that psychological

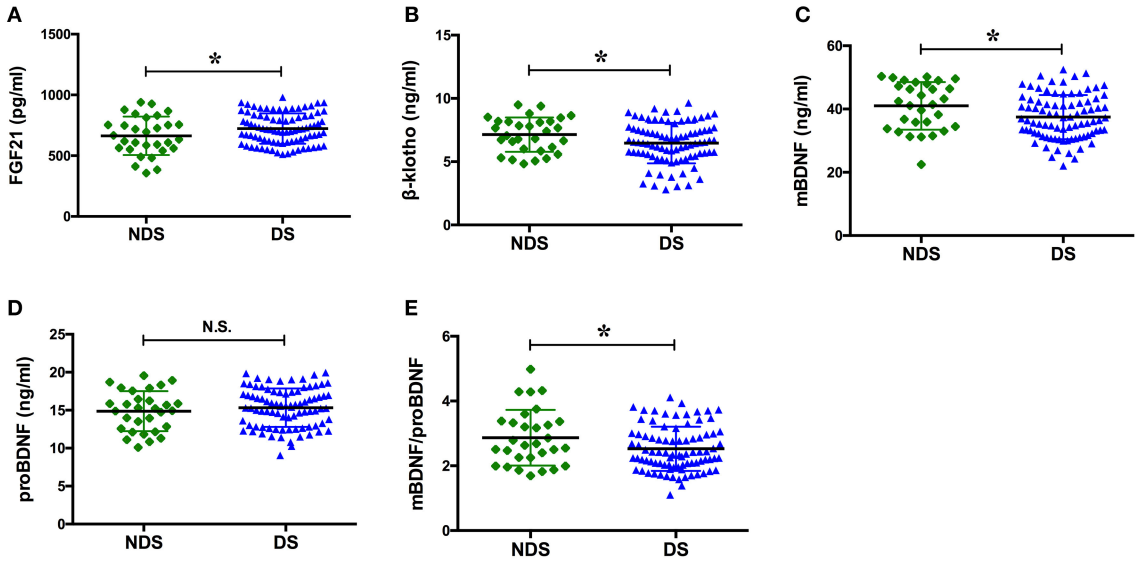

FIGURE 2 | Serum levels of the factors in NDS and DS. BDNF, brain derived neurotrophic factor; DS, depressive symptoms; FGF21, fibroblast growth factor 21; NDS, no depressive symptoms; N.S., no significance. The means of the two groups were compared using a *t*-test. The serum concentration of FGF21 in the group with DS was significantly greater than that observed in the group with NDS [(723.23 ± 125.49) pg/ml vs. (663.58 ± 157.78) pg/ml, $p = 0.039$], while the serum concentrations of β-klotho, mBDNF, and mBDNF/proBDNF were significantly lower [(6.46 ± 1.60) ng/ml vs. (7.14 ± 1.35) ng/ml, $p = 0.041$; (37.47 ± 6.98) ng/ml vs. (41.00 ± 7.50) ng/ml, $p = 0.021$; and (2.53 ± 0.68) vs. (2.86 ± 0.86), $p = 0.029$, respectively]. *$p < 0.05$.

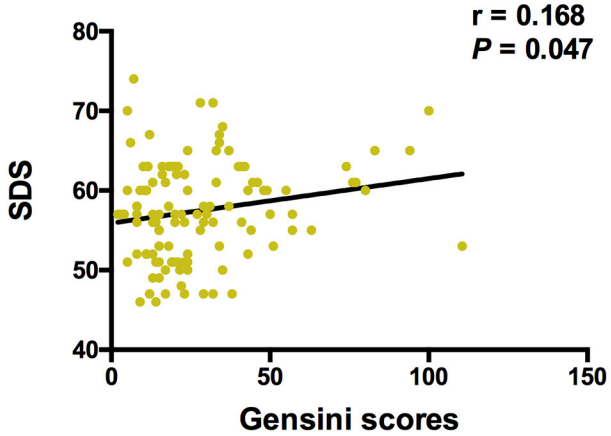

FIGURE 3 | Spearman correlation analysis between Gensini scores and SDS. SDS, self-rating depression scale. The correlation analysis was performed using the Spearman correlation analysis. There is a positive correlation between the Gensini scores and the SDS scores, and the correlation coefficient (r) was 0.168 ($p = 0.047$).

intervention is necessary for patients with CHD. The Sertraline Antidepressant Heart Attack Randomized Trial found that patients with acute coronary syndrome and comorbid depression exhibited greater levels of platelet factor 4, platelet endothelial cell adhesion molecule-1, and thromboxane, which suggests enhanced platelet activation and aggregation. Platelets interact with leukocytes to stimulate cytokine release and to promote vascular intimal injury and atherosclerosis development. This process increases the incidence of CHD in healthy people, accelerates atherosclerosis, and increases mortality in patients with CHD (36, 37). Furthermore, the concentrations of interleukin-6, C-reactive protein, and tumor necrosis factor-α are increased in patients with CHD with co-morbid depression (38, 39). Therefore, the role of inflammatory processes in the physiological mechanisms of CHD with co-morbid depression has received much attention. In addition, depression-related hypothalamic-pituitary-adrenal axis hyperactivity could increase sympathetic excitability and catecholamine secretion, which leads to excessive vasoconstriction and coronary artery spasm and further aggravates myocardial ischemia (40, 41). In parallel, a decrease in the synthesis of platelet- and endothelial cell-derived nitric oxide in depressive patients limits vasodilation (42), which may exacerbate atherosclerotic plaque formation. Based on these observations, it is likely that depression and coronary atherosclerosis co-exist and interact with each other to affect the quality of life and patient prognosis.

The findings of the present study showed that patients with SCAD suffer from a variable degree of depression. The depression score is positively correlated with the severity of coronary artery stenosis. In this regard, psychological assessment should be adopted for patients with cardiac disease, especially in patients with severe coronary artery stenosis. Therefore, appropriate antidepressant drugs and active psychotherapy are necessary for symptomatic management.

Although FGF21 is a hormone-like endocrine factor that is mainly secreted by the liver, numerous studies have shown that FGF21 acts as an insulin-like growth factor to improve

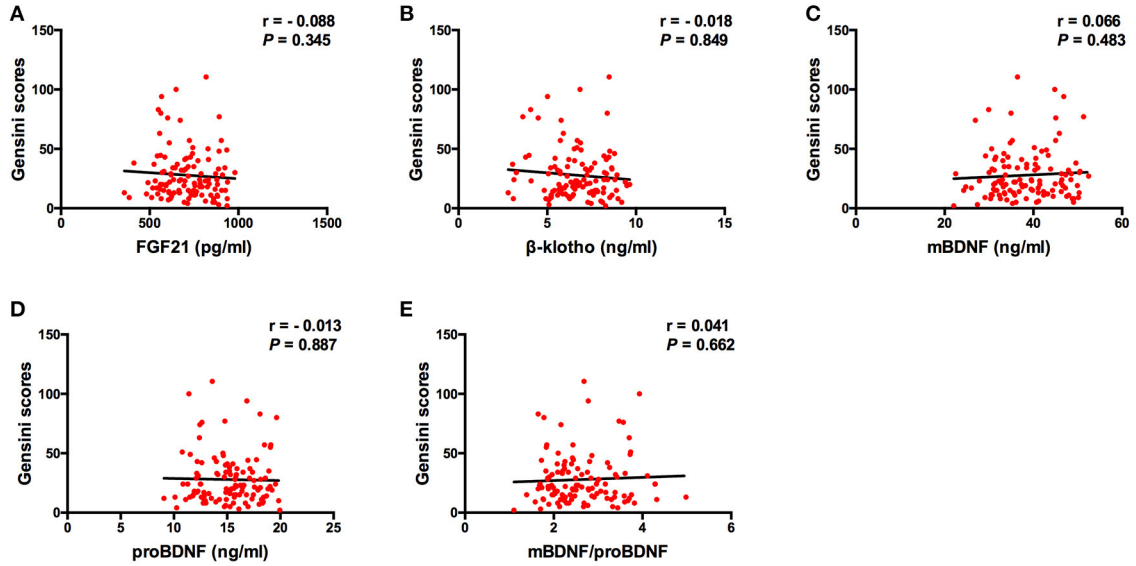

FIGURE 4 | Spearman correlation analysis between serum levels of the factors and Gensini scores. BDNF, brain derived neurotrophic factor; FGF21, fibroblast growth factor 21. The correlation analysis was performed using the Spearman correlation analysis. There was no significant correlation between FGF21, β-klotho, mBDNF, proBDNF, and mBDNF/proBDNF concentrations, and the Gensini scores.

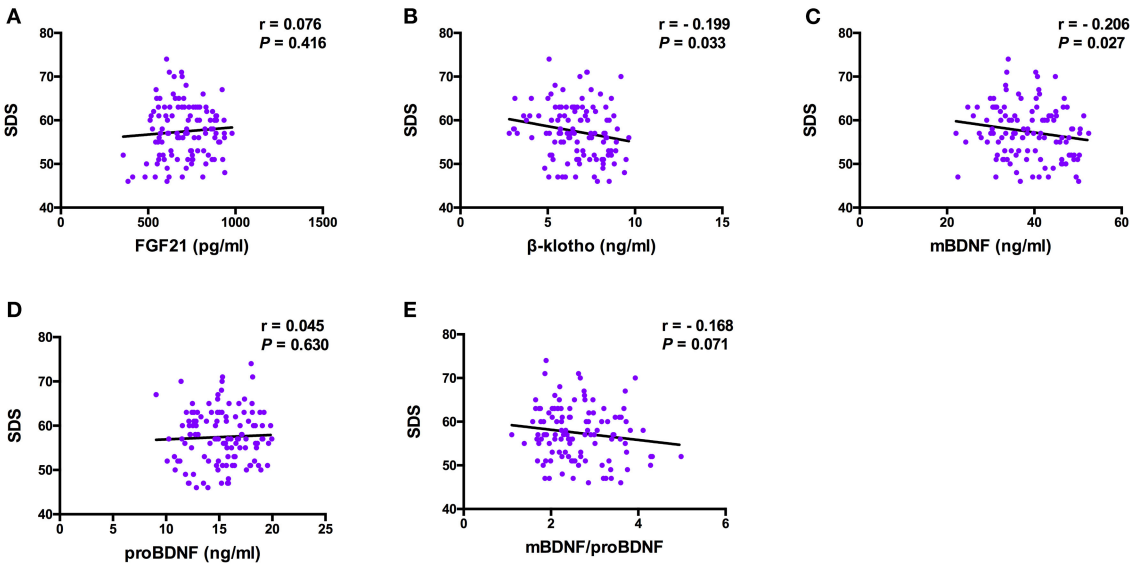

FIGURE 5 | Pearson correlation analysis between serum levels of the factors and SDS. BDNF, brain derived neurotrophic factor; FGF21, fibroblast growth factor 21; SDS, self-rating depression scale. The correlation analysis was performed using the Pearson correlation analysis. β-klotho and mBDNF levels were negatively correlated with the SDS scores ($r = -0.199$, $p = 0.033$; and $r = -0.206$, $p = 0.027$, respectively).

glucose metabolism, which would, in turn, improve serum insulin sensitivity and glucose clearance (43, 44). Additionally, FGF21 inhibits sterol regulatory element-binding protein-1 and activates uncoupling protein-1/2. In doing so, FGF21 reduces the expression of genes associated with fatty acid synthesis and fat utilization (44, 45). After virtual histology-intravascular ultrasound and FGF21 detection in 68 patients with CHD, serum FGF21 levels were significantly positively correlated with the atherosclerotic plaque burden, which can be defined as (plaque + media)/external elastic membrane (46). Unlike previous studies, we observed that patients in the SCAD group demonstrated a lower serum FGF21 compared with the HC group, indicating that FGF21 did not protect against SCAD in this study.

TABLE 3 | Multiple linear regression analysis between serum levels of the factors and Gensini scores.

Parameters	*B*	SE	95% CI	*t*	*P*-value
FGF21	−0.012	0.015	−0.042∼0.017	−0.818	0.415
β-klotho	−1.257	1.334	−3.901∼1.387	−0.942	0.348
mBDNF	0.167	0.283	−0.393∼0.728	0.591	0.555
proBDNF	−0.103	0.788	−1.664∼1.458	−0.131	0.896

BDNF, brain derived neurotrophic factor; FGF21, fibroblast growth factor 21. The multiple linear regression analysis showed that there was no significant correlation between FGF21, β-klotho, mBDNF, and proBDNF concentrations, and the Gensini scores ($F = 0.576$, $p = 0.681$, $R^2 = 0.020$, $B0 = 40.067$), and none of the variables were significant predictors of Gensini score ($p > 0.05$).

TABLE 4 | Multiple linear regression analysis between serum levels of the factors and SDS.

Parameters	*B*	*SE*	95% CI	*t*	*P*-value
FGF21	0.005	0.004	−0.003∼0.014	1.240	0.217
β-klotho	−0.772	0.375	−1.515∼0.029	−2.059	0.042
mBDNF	−0.140	0.079	−0.298∼0.017	−1.763	0.081
proBDNF	0.031	0.013	−0.408∼0.470	0.140	0.889

BDNF, brain derived neurotrophic factor; FGF21, fibroblast growth factor 21. The multiple linear regression analysis built a significant model to predict SDS score based on the serum levels of those factors ($F = 2.492$, $p = 0.047$, $B0 = 63.764$), but the coefficient of determination was low ($R^2 = 0.082$). Analysis of variance inflation factors (VIFs) did not demonstrate multicollinearity between factors. No violations of linearity were detected. Significant predictor for a higher SDS score was the lower β-klotho levels serum ($B = -0.772$, $p = 0.042$).

TABLE 5 | Incidence of depressive symptoms in SCAD patients with low or high FGF21, β-klotho, mBDNF, and proBDNF levels.

	Without depressive symptoms ($n = 30$)	with depressive symptoms ($n = 86$)	χ^2	*P*-value
FGF21				
Low level	23	61	0.366	0.545
High level	7	25		
β-klotho				
Low level	9	39	2.160	0.142
High level	21	47		
mBDNF				
Low level	17	67	5.023	0.025
High level	13	19		
proBDNF				
Low level	9	23	0.118	0.731
High level	21	63		

BDNF, brain derived neurotrophic factor; FGF21, fibroblast growth factor 21; SCAD, stable coronary artery disease. A chi-squared test was used.

To exert its physiological function, FGF21 interacts with the co-receptor, β-klotho. The FGF21/β-klotho complex then binds to the plasma membrane-localized FGF receptor and activates the extracellular signal-regulated kinase 1/2 and receptor tyrosine kinases to mediate signaling cascades (47, 48). Therefore, β-klotho is the basis for the tissue-specific expression and biological function of FGF21. As an anti-aging protein, β-klotho can delay senescence through various mechanisms, including anti-oxidation, anti-senescence, and anti-autophagy. β-klotho also regulates a number of signaling pathways, including insulin-like growth factor and Wnt pathways (49). Research suggests that β-klotho plays an important role in the progression of age-related diseases such as Alzheimer's disease and neurodegeneration (50, 51). Interestingly, age is a risk factor for DS. Depression, as a heterogeneous disorder (10, 52, 53), may be associated with impaired function of various organs caused by aging (e.g., thalamic dysfunction, which leads to emotional instability) (54). In this study, serum β-klotho was significantly decreased in the group with DS. β-klotho was significantly negatively correlated with the SDS scores. To the best of our knowledge, this is the first study to demonstrate the role of β-klotho in CHD with co-morbid depression. Further detailed studies are necessary.

As a member of the neurotrophin family, BDNF is widely expressed in the adult mammalian brain in close proximity to its receptor, tropomyosin-related kinase B (TrkB). BDNF-TrkB signaling activates downstream effectors such as mitogen-activated protein kinase and phosphatidylinositol 3-kinase, thereby eliciting a protective effect on neurons (55–57). Recent studies have shown that BDNF-TrkB signaling is important in the survival of vascular endothelial cells and may promote proliferation and migration of endothelial cells in ischemic regions (28, 29, 58). In addition, exercise can promote myocardial angiogenesis in mice with myocardial infarction by activating the BDNF-TrkB axis. This pathway improves left ventricular function and has a beneficial effect on cardiac function (59, 60). Unlike mBDNF, proBDNF has a high affinity for p75NTR. ProBDNF-p75NTR activates the JNK pathway to upregulate p53 expression and initiate apoptosis. ProBDNF-p75NTR also interferes with neurotransmitter release and inhibits axonal outgrowth (61). A series of reports demonstrated that serum BDNF in depressive patients is significantly lower than that observed in the healthy population (62–64). However, few studies report the expression of mBDNF and proBDNF in patients with SCAD with co-morbid depression. Our research group previously reported that serum mBDNF was decreased in patients with SCAD, and the group with DS demonstrated significantly lower concentrations of mBDNF and mBDNF/proBDNF. Additionally, there was a negative correlation between serum mBDNF and the SDS scores. The patients in the lower mBDNF group had a higher incidence of DS than the higher level group, suggesting that a greater serum mBDNF may help to improving depression.

There are some limitations to the present study. First, DS was assessed only by using SDS. A history of depression or other psychiatric disorders was assessed by interviews, but not by professional diagnostic criteria or psychiatric examinations, which may affect the reliability of the results. Second, this study did not discuss residual confounding factors that may be relevant to the research, such as education, income, social support, and marital status. Third, owing to the short duration of hospitalization and limited maneuverability, this study was

not able to determine whether psychotherapy could improve the quality of life for patients. Thus, future work is necessary to clarify these considerations. Finally, this study was a cross-sectional study with small sample size. Therefore, future population-based studies will be necessary to confirm the results.

CONCLUSION

In this study, a decrease in serum FGF21 wae closely related to SCAD. Lower serum β-klotho, mBDNF, and proBDNF might indicate the development of DS in patients with SCAD and might thus represent potential diagnostic and/or therapeutic targets for patients suffering from SCAD and co-morbid depression. Hence, early recognition of abnormalities in FGF21, β-klotho, mBDNF, and proBDNF levels is crucial. Effective strategies should be formulated to improve DS and prognosis for patients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This study was approved by the Ethics Committee of the Third Affiliated Hospital of Soochow University and was registered in the Chinese Clinical Trial Registry (ChiCTR1900020594). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LY and LZ conceived the study and participated in the design. YW, ZC, JD, and KH participated in the design, collected the data, performed statistical analyses, and drafted the manuscript. YW, ZC, and BZ conducted the analysis and developed the figures. LZ, YW, and ZC revised the manuscript. All authors read 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/fpsyt.2020.587492/full#supplementary-material

Supplementary Figure 1 | Dendrogram of the cluster analysis of fibroblast growth factor 21.

Supplementary Figure 2 | Dendrogram of the cluster analysis of β-klotho.

Supplementary Figure 3 | Dendrogram of the cluster analysis of mature brain-derived neurotrophic factor.

Supplementary Figure 4 | Dendrogram of the cluster analysis of brain-derived neurotrophic factor precursor.

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GENERAL COMMENTARY
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Commentary: Serum Biomarkers Are Potential Diagnosis and Treatment Targets for Depressive Symptoms in Patients With Cardiovascular Diseases

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A Commentary on

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Cardiovascular diseases (CVDs) and depression are the major causes for death and disability worldwide (1, 2). Currently, depression has been confirmed as an independent risk factor for CVDs (3). However, the comorbidity of CVDs and depression still lacks effective objective diagnostic indicators. This is the question that Wu et al. (4) tried to answer in this issue of *Frontiers in Psychiatry* (*Interplay of Stress, Pain and Psychiatric Diseases*). The authors recruited 116 patients with stable coronary artery disease (SCAD) and 45 healthy controls. According to the Zung Self-Rating Depression Scale (SDS), the SCAD patients were divided into a depressive group and a non-depressive group, and measured serum levels of fibroblast growth factor 21 (FGF21), β-klotho, mature brain-derived neurotrophic factor (mBDNF), and BDNF precursor (BDNF precursor, proBDNF). The findings showed that depression score was positively correlated with the severity of SCAD, and that serum FGF21, β-klotho, mBDNF, and proBDNF levels were linked to depressive symptoms in SCAD patients.

As an available approach to diagnose depression, clinical face-to-face interviews still have the following problems. Firstly, to facilitate comparison, physicians try to promote specific tools to evaluate depression in patients with CVDs. Still, many different questionnaires are adopted in a large number of various studies to screen patients. Differential standards for selecting evaluation scales may result in an evitable bias in results and conclusions. Secondly, depressive symptoms in patients with CVDs are often firstly recognized by the cardiologists. Unfortunately, some cardiologists have not yet been professionally trained in psychiatry. The non-professional background may affect the efficacy of scale evaluation. Thirdly, a few patients with CVDs are severely ill at hospital admissions, for example with acute myocardial infarction, acute heart failure, and even multiple organ failure. It seems difficult to develop a questionnaire for these patients. Therefore, it is necessary to find objective detection indicators, such as serum biomarkers, to assess the relationship between CVDs and depression comorbidities, or the severity of depressive symptoms in CVDs patients.

FGF21 is a member of the FGF family and is expressed in multiple organs, including liver, kidney, fat cells, and cardiomyocytes. FGF21 can specifically bind to the co-receptor

β-klotho and release into the circulation (5), and the levels of FGF21 are significantly affected by β-klotho (6). BDNF is an essential member of the neurotrophic factor family, regulating the development and plasticity of neurons. Notably, BDNF as a clue to explore potential biomarkers of cardiovascular diseases comorbid depression has been reported (4, 7). Plasminogen activator inhibitor-1 (PAI-1) could act as a mediator of depression-induced CVDs through sleep disorder, adiposity, BDNF metabolism, systemic inflammation, and hypothalamic-pituitary-adrenal (HPA) axis dysregulation. Similarly, sigma-1 receptor chaperone plays a vital role in CVDs comorbid with depression (8). Sigma-1 receptor agonists such as endogenous neurosteroid dehydroepiandosterone (DHEA) and selective serotonin reuptake inhibitors (SSRIs) have shown potent cardio-protective and antidepressant effects. In this study, Wu et al. (4) firstly found that serum β-klotho in the depression group was significantly reduced, and that β-klotho was negatively correlated with the depression score. However, the detailed mechanisms remain to be identified. Also, Wu et al. (4) found that serum mBDNF levels were decreased in SCAD patients, and that mBDNF level and mBDNF/proBDNF ratio in the depression group were significantly decreased. More importantly, serum mBDNF levels were negatively correlated with SDS score. Subgroup analysis found that the incidence of depression in the low-level mBDNF group was higher than that of the high-level mBDNF group, suggesting that increasing serum mBDNF levels may improve the depression symptoms of SCAD patients. However, there are several limitations in the study. The study did not discuss confounding factors that might affect the results of the study, such as education, income, social support, and marital status. In addition, this is a phenomenological study that only observed the differences of these biomarkers in the SCAD and SCAD with depressive symptoms groups. The authors did not delve into the possible mechanisms underlying the differences in biomarkers. Furthermore, the authors did not discuss the sensitivity and specificity of these biomarkers for the diagnosis of depressive symptoms in patients with CVDs.

Sir William Harvey, 350 years ago, first observed that negative emotions have a deleterious effect on the heart (9). Since the late 1980s, research directions have gradually shifted from psychiatric patients with depression to the high-risk factors for depression in patients diagnosed with CVDs in the community, aiming to explore the impact of depression on CVDs (10). Unfortunately, most studies still maintain "depression" and "CVDs" as two separate diseases, lacking an accurate definition for "psycho-cardiology." Therefore, it is difficult for doctors to distinguish the sequence of the occurrence and development of depression and CVDs, and to study the causal relationship between depression and CVDs. Another difficulty is that the mechanisms underlying the co-morbidity of CVDs and depression are complex and still not fully understood. In addition to traditional mechanisms, novel mechanisms such as gut microbiota, endocrine signaling, microRNAs have also been extensively studied (11). This might propose additional challenges to understand the pathogenesis and therapeutic mechanisms of the comorbidity. In addition to potential confounding factors, including smoking, diabetes, obesity, and sedentary lifestyle, further long-term studies are needed to track the relationship between CVDs, mood, depression, and psychosocial status. Considering the different focus of diagnosis and treatment by different specialists, diagnosing and treating such patients in a multidisciplinary manner is necessary. Moreover, reliable testing indicators, such as serum biomarkers, need to be established to evaluate depressive symptoms and CVDs and the diagnosis, treatment, and prognosis of the comorbid patients. Besides, it seems inappropriate for several studies to simply classify patients as depressed or non-depressed through the depression scale. Evidence has shown a correlation between the severity of depression and cardiac risk, and even the appearance of mild symptoms will bring risks (12). However, patients with mild depressive symptoms may be classified as non-depressed based on the widely used depression questionnaires. Consequently, artificial categorization of depression severity scores (for example, non-depressed vs. depressed) may weaken the strength of the correlation between depression severity and cardiac prognosis.

In summary, depression as one of the independent cardiovascular risk factors has a significant impact on the public health. The paper by Wu et al. (4) has raised the necessary attention to seek biomarkers for CVDs and depression comorbidity. Future studies should better understand the bidirectional relationship between CVDs and depression and design more rigorous long-term follow-up research programs to find effective biomarkers in a larger sample.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Neurotensin and Xenin Show Positive Correlations With Perceived Stress, Anxiety, Depressiveness and Eating Disorder Symptoms in Female Obese Patients

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Objective: Neurotensin and xenin are two closely related anorexigenic neuropeptides synthesized in the small intestine that exert diverse peripheral and central functions. Both act via the neurotensin-1-receptor. In animal models of obesity reduced central concentrations of these peptides have been found. Dysregulations of the acute and chronic stress response are associated with development and maintenance of obesity. Until now, associations of both peptides with stress, anxiety, depressiveness, and eating disorder symptoms have not been investigated. The aim of the present study was to examine associations of neurotensin and xenin with these psychological characteristics under conditions of obesity.

Materials and Methods: From 2010 to 2016 we consecutively enrolled 160 inpatients (63 men and 97 women), admitted due to obesity and its mental and somatic comorbidities. Blood withdrawal und psychometric tests (PSQ-20, GAD-7, PHQ-9, and EDI-2) occurred within one week after admission. We measured levels of neurotensin and xenin in plasma by ELISA.

Results: Mean body mass index was 47.2 ± 9.5 kg/m^2. Concentrations of neurotensin and xenin positively correlated with each other (women: $r = 0.788$, $p < 0.001$; men: $r = 0.731$, $p < 0.001$) and did not significantly differ between sexes ($p > 0.05$). Women generally displayed higher psychometric values than men (PSQ-20: 58.2 ± 21.7 vs. 47.0 ± 20.8, $p = 0.002$; GAD-7: 9.7 ± 5.8 vs. 7.1 ± 5.3, $p = 0.004$; PHQ-9: 11.6 ± 6.6 vs. 8.8 ± 5.9, $p = 0.008$; EDI-2: 50.5 ± 12.8 vs. 39.7 ± 11.9, $p < 0.001$). Only women showed positive correlations of both neuropeptides with stress (neurotensin: $r = 0.231$, $p = 0.023$; xenin: $r = 0.254$, $p = 0.013$), anxiety (neurotensin: $r = 0.265$, $p = 0.009$; xenin: $r = 0.257$, $p = 0.012$), depressiveness (neurotensin: $r = 0.281$, $p = 0.006$; xenin: $r = 0.241$, $p = 0.019$) and eating disorder symptoms (neurotensin: $r = 0.276$, $p = 0.007$; xenin: $r = 0.26$, $p = 0.011$), whereas, men did not ($p > 0.05$).

Conclusion: Neurotensin and xenin plasma levels of female obese patients are positively correlated with perceived stress, anxiety, depressiveness, and eating disorder symptoms. These associations could be influenced by higher prevalence of mental disorders in women and by sex hormones. In men, no correlations were observed, which points toward a sex-dependent regulation.

Keywords: gut-brain axis, obesity, psychiatric, psychosomatic, sex difference

HIGHLIGHTS

- Neurotensin and xenin are long-known neuropeptides with anorexigenic effects.
- Neurotensin and xenin seem to be associated with psychopathology under conditions of obesity.
- Sex-specific regulation of neurotensin and xenin was found for perceived stress and anxiety.
- Only in obese women neurotensin and xenin showed positive correlations with depressiveness and eating disorder symptoms.
- Alterations of circulating levels of neurotensin and xenin may be involved in the emergence and maintenance of obesity.

INTRODUCTION

With a worldwide prevalence of 13.1%, which has increased threefold since 1975, obesity (body mass index, BMI $\geq$ 30 kg/m^2) is a widespread and serious disease, mostly because of its physical and mental comorbidities (World Health Organization, 2017, 2020). In Europe, 23.3% of the adult population is obese (World Health Organization, 2017). The prevalence of 38.2% in the United States of America is critically high among adults (Organisation for Economic Co-operation and Development, 2017). Comorbidities of obesity include dyslipidemia and insulin resistance together with manifest diabetes mellitus type 2 and arterial hypertension (Grundy, 2002; Garg et al., 2014). In addition, mental comorbidities are associated with obesity (Petry et al., 2008; Preiss et al., 2013; Herpertz, 2015). Depressive, anxiety, somatoform, and binge eating disorders (BED) are the most frequent ones (Petry et al., 2008; Preiss et al., 2013; Herpertz, 2015). Moreover, psychosocial risk factors are involved in the etiology of obesity and obesity itself is a risk factor of major depression (Herpertz, 2015).

Furthermore, increased levels of perceived acute and chronic stress along with dysregulations of the endocrine stress response likely contribute to the genesis and maintenance of obesity (Morris et al., 2015). Chronic stress is biologically reflected by enduring stimulation of the hypothalamic-pituitary-adrenal (HPA) axis leading to elevated levels of circulating glucocorticoids (Strack et al., 1995; Morris et al., 2015). Subsequent catabolic effects and the impact on energy balance in terms of orexigenic signaling (e.g., via neuropeptide Y, NPY) further contribute to the maintenance of obesity and its comorbidities (Strack et al., 1995; Chrousos, 2009). Moreover, a pro-inflammatory state due to large amounts of endocrinely active visceral fat tissue, which is another stimulus of the HPA axis, and increased stress levels are in close interaction (Nezi et al., 2000; Tilg and Moschen, 2006). An important connection of this psychobiological relationship is the bidirectional gut-brain axis (Lee and Abizaid, 2014). In this crosstalk, intestinally and centrally secreted peptides are crucial (Holzer and Farzi, 2014; Lach et al., 2018). Long-term regulation of energy balance and body weight is redundantly regulated by several important hormones, e.g., insulin, leptin, and ghrelin (Pape, 2014). Therefore, dysregulation of peptides of the gut-brain axis are in the focus of obesity research (Beck, 2000; Boughton and Murphy, 2013). Nevertheless, several potentially relevant peptides have not yet been investigated in this context.

Peptides of the highly conserved neurotensin/xenopsin/xenin family exert diverse peripheral and central functions along the gut-brain axis (Feurle, 1998). A central anorexigenic effect by neurotensin and xenin was shown in rats, whereas, this effect has not been shown yet for the amphibian peptide xenopsin (Araki et al., 1975; Luttinger et al., 1982; Alexiou et al., 1998; McConn et al., 2015).

Neurotensin, a tridecapeptide, was detected in bovine hypothalamus (Carraway and Leeman, 1973). It is derived from the same precursor protein as neuromedin N (NMN), secreted in the central nervous system (CNS) and peripherally by N-cells of the intestinal mucosa due to postprandially increased free fatty acids (Kislauskis et al., 1988; Malendowicz, 1998; Schroeder and Leinninger, 2018). Important brain areas of neurotensin secretion are amygdala, hippocampus, ventral tegmental area (VTA), arcuate, and paraventricular nuclei (St-Gelais et al., 2006). Besides the anti-hypertensive effect of the peptide, many other effects were discovered (Blackburn, 1978) encompassing a stimulation of gut motility and pancreatic secretion of glucagon and insulin, a reduction of gastric acid secretion and a role as growth factor (Blackburn et al., 1980; Yawata et al., 1984; Carraway and Plona, 2006; Kalafatakis and Triantafyllou, 2011). Central effects of neurotensin are hypothermia, food intake suppression, and opioid-independent analgesia as well as an endogenous antipsychotic activity because of interactions with the dopaminergic system (Martin et al., 1980; Luttinger et al., 1982; Furuta et al., 1984; Fuxe et al., 1992). Furthermore, neurotensin displays regulatory functions in pituitary secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Herbison and Theodosis, 1991, 1992). Four different receptors are responsible for the physiological effects of neurotensin, especially the high-affinity neurotensin-receptor-1 (NTS_1) and low-affinity neurotensin-receptor-2 (NTS_2), which are G-protein-coupled-receptors (GPCRs) (Tanaka et al., 1990;

Chalon et al., 1996; Mazella et al., 1998; Jacobsen et al., 2001).

Xenin, was discovered in 1992 but is still relatively little studied (Feurle et al., 1992). The 25 amino acid peptide hormone is released by enteroendocrine K-cells of the duodenum (Anlauf et al., 2000). The same cells synthesize glucose-dependent insulinotropic peptide (GIP) whose functions are closely related to the peripheral effects of xenin (Anlauf et al., 2000; Cho and Kieffer, 2010). Despite identical cells of origin in the duodenum, no sequence homology exists between the 42-amino acid polypeptide GIP and xenin (Feurle, 1998; Yip and Wolfe, 2000). GIP binds to the glucose-inhibitory peptide receptor, whereas, xenin primarily acts via the NTS_1 (Clemens et al., 1997; Baggio and Drucker, 2007; Kim et al., 2016).

As observed for neurotensin, peripheral xenin is primarily released postprandially inducing a delayed gastric emptying rate, contraction of gall bladder, lipolysis of white adipose tissue, and stimulation of exocrine and endocrine pancreatic functions associated with an antidiabetic effect (Feurle et al., 1992; Kamiyama et al., 2007; Kim and Mizuno, 2010a; Wice et al., 2012; Bhavya et al., 2018). In the CNS, xenin suppresses appetite through effects on several food intake-regulatory centers in the hypothalamus and brainstem independent of melanocortin and leptin signaling pathways (Nandar et al., 2008; Leckstrom et al., 2009; Kim and Mizuno, 2010a). Xenin and neurotensin show 20% sequence homology (five amino acids in identical position), especially near the C-terminus (Yip and Wolfe, 2000).

Due to their physiological effects, both neurotensin and xenin have been examined in the context of obesity and in relation to somatic sequelae of the disease (Craig et al., 2018; Schroeder and Leinninger, 2018). Antidiabetic properties of xenin and dysregulations of neurotensin and pro-neurotensin in obesity and high-fat diet are current areas of interest (Weiss et al., 2001; Wice et al., 2010; Gault et al., 2015; Li et al., 2016; Hasib et al., 2017). Moreover, anorexigenic effects of both peptides have been examined, because in animal models of obesity, decreased central concentrations of neurotensin have been detected together with reduced food intake after intracerebroventricular (icv) administration of xenin and neurotensin in rodents (Beck et al., 1990; Williams et al., 1991a; Cooke et al., 2009). Until now, mental conditions of obese patients and xenin levels have not been investigated.

The aim of the present study was to investigate potential associations between plasma levels of neurotensin and xenin and perceived stress, anxiety, depressiveness, and eating disorder symptoms in obese inpatients. Women and men were examined separately since expression of neurotensin and were shown to be sex-dependent (Herbison and Theodosis, 1991, 1992; Alexander, 1993).

MATERIALS AND METHODS

Participants

Investigations were conducted according to the Declaration of Helsinki. All participants gave written informed consent and the study was approved by the institutional ethics committee of the Charité – Universitätsmedizin Berlin (protocol number: EA1/130/16).

In the period between September 2010 and December 2016 we consecutively recruited 160 obese inpatients (97 women and 63 men). All participants were hospitalized in the Department of Psychosomatic Medicine Charité – Universitätsmedizin Berlin, where they received therapy due to obesity and its mental and somatic comorbidities. Inclusion criteria encompassed a BMI $\geq$ 30 kg/m^2 and a minimum age of 18 years. Current pregnancy or lactation period, dialysis, hypercortisolism, malignoma, psychotic disorders as well as treatment with immunomodulatory drugs (e.g., methotrexate, azathioprine, and oral corticosteroids) were exclusion criteria. Furthermore, patients with somatic or somatoform disorders of the gastrointestinal tract or after surgery of the gastrointestinal tract including bariatric operations, except for appendectomy, and uncomplicated cholecystectomy, were excluded.

Procedure

Study enrolment including clarification of potential exclusion criteria and blood withdrawal was conducted within four days after admission. Venous blood sample from a forearm vein of overnight fasted patients was taken every Friday between 7.00 am and 8.00 am. It was permitted to drink a small amount of water, but patients were not allowed to drink coffee or exercise before blood collection. Patients were also advised not to smoke prior to blood withdrawal. At the same morning we assessed actual medication, body height and fasting weight of participants in light underwear to calculate the BMI.

Psychometric Measurements

Associated psychometric data collection occurred no more than two days before or five days after blood withdrawal. The following self-reported questionnaires were completed by the participants.

The Perceived Stress Questionnaire (PSQ) was used to evaluate the severity of subjective stress perception (Levenstein et al., 1993) with the shortened 20-item version (PSQ-20) by Fliege et al. (2005). The questionnaire encompasses the subscales "worries," "tension," "joy" with relation to the stress response and "demands" regarding the perception of external stressors (Levenstein et al., 1993). Scores ranged from zero to 100. Crohnbach's alpha for the current sample was 0.892, 0.904, 0.824, and 0.838 for the four subscales, respectively, and 0.872 for the total score.

To evaluate the levels of anxiety and depression, we used two subscales of the Patient Health Questionnaire (PHQ) (Spitzer et al., 1999). For the assessment of anxiety, we used the German version (Löwe et al., 2008) of the Generalized Anxiety Disorder-7 (GAD-7) scale with seven items (4-point Likert scale with a range from zero up to three) as an established and widely used tool (Spitzer et al., 2006). Crohnbach's alpha for the current sample was 0.903. The PHQ-9 with total scores from zero to 27 was designed to measure the severity of depressive symptoms and consists of nine items (Spitzer et al., 1999). We employed the German version of the subscale (Löwe et al., 2002). Crohnbach's alpha for the current sample was 0.894.

The severity of eating disorder symptoms was measured by the Eating Disorder Inventory (EDI), which consists of 64 items (Garner et al., 1983). EDI comprises eight subscales covering different aspects of eating disorders: "drive for thinness," "bulimia," "body dissatisfaction," "ineffectiveness," "perfectionism," "interpersonal distrust," "interoceptive awareness," and "maturity fears." In detail, the German version of the EDI-2, a revised version of the EDI with three additional subscales (which were not applied in the current study) was used. Scores range from zero to 100 (Garner, 1991; Thiel et al., 1997). Crohnbach's alpha for the current sample was 0.795, 0.896, 0.856, 0.901, 0.748, 0.784, 0.872, and 0.690 for the eight subscales, respectively, and 0.809 for the total score.

Laboratory Analysis

Venous blood samples were collected in chilled standard laboratory EDTA tubes containing aprotinin (1.2 Trypsin Inhibitory Unit per 1 ml blood; ICN Pharmaceuticals, Costa Mesa, CA, United States) as peptidase inhibitor and stored on ice immediately after withdrawal. EDTA tubes were then centrifuged at 4°C for 10 min at 3000 *g*. Blood plasma of each sample was separated and stored at −80°C. Neurotensin and xenin were measured by the use of enzyme-linked immunosorbent assays (ELISA, neurotensin: catalog # EK-048-03, xenin: catalog # EK-046-74, Phoenix Pharmaceuticals Inc., Burlingame, CA, United States, 0% cross reactivity between both analytes according to manufacturer's information). For neurotensin, the intra-assay variability was 5.8% and for xenin 7.3%. Every measurement was conducted twice, whereof we calculated the mean value.

Statistical Analysis

Kolmogorov-Smirnov test was used to determine the distribution of the data. Because all data were normally distributed, we employed *t*-tests and chi-square tests analyzing differences between two groups. Effect sizes were estimated by Cohen's *d* (*t*-test), Phi-coefficient φ (chi-square test) or Cramér's *V* (chi-square test). Correlations were assessed using Pearson's analysis. The correlations and differences between groups were considered significant when $p < 0.05$. Due to the explorative character of the study no *p* adjustment has been performed. Multivariable linear regression was used to investigate the effect of age, gender, various comorbidities and medication. Data are expressed as mean ± standard derivation (SD) and statistical analyses were executed using SigmaStat 3.1 (Systat Software, San Jose, CA, United States) and IBM SPSS Statistics Version 26.0.0.0 (IBM corp, Armonk, NY, United States).

RESULTS

Characterization of the Study Population

Anthropometric, endocrine and psychometric characteristics of the study population ($n = 160$) and a comparison between women and men are presented in **Table 1**. Mean BMI was 47.2 ± 9.5 kg/m^2 (range: 31.8–75.1 kg/m^2) and mean age 45.5 ± 13.7 years (range: 19–73 years). PSQ-20 data was missing

TABLE 1 | Anthropometric, psychometric, and endocrine characteristics of study populations.

Parameter	Men (n = 63)	Women (n = 97)	p	d
Age (years)	47.5 ± 13.6 (19–73)	44.2 ± 13.5 (20–73)	0.131	0.246
BMI (kg/m^2)	45.5 ± 8.2 (32–67)	48.4 ± 10.0 (33–75)	0.059	0.307
PSQ-20 total score	47.0 ± 20.8 (3–95)	58.2 ± 21.7 (2–98)	**0.002**	0.521
- Worries	42.6 ± 24.9 (0–100)	59.1 ± 27.2 (0–100)	**<0.001**	0.624
- Tension	50.2 ± 26.7 (0–100)	61.6 ± 27.1 (0–100)	**0.011**	0.420
- Joy	42.5 ± 22.9 (0–100)	37.7 ± 24.0 (0–100)	0.214	0.203
- Demands	37.5 ± 25.2 (0–100)	49.6 ± 23.9 (0–100)	**0.003**	0.493
GAD-7 total score	7.1 ± 5.3 (0–20)	9.7 ± 5.8 (0–21)	**0.004**	0.469
PHQ-9 total score	8.8 ± 5.9 (0–25)	11.6 ± 6.6 (1–26)	**0.008**	0.436
EDI-2 total score	39.7 ± 11.9 (19–72)	50.5 ± 12.8 (27–84)	**<0.001**	0.866
- Drive for thinness	50.1 ± 21.6 (9–97)	61.3 ± 20.0 (17–97)	**0.001**	0.541
- Bulimia	22.2 ± 19.3 (0–74)	29.6 ± 24.4 (0–97)	**0.046**	0.327
- Body dissatisfaction	71.5 ± 21.7 (33–100)	89.4 ± 13.7 (46–100)	**<0.001**	1.030
- Ineffectiveness	31.1 ± 18.2 (0–78)	46.2 ± 21.0 (6–90)	**<0.001**	0.751
- Perfectionism	38.6 ± 19.0 (7–87)	43.2 ± 20.4 (7–93)	0.155	0.232
- Interpersonal distrust	40.3 ± 17.8 (0–80)	47.4 ± 17.4 (9–89)	**0.013**	0.405
- Interoceptive awareness	26.4 ± 18.2 (0–82)	39.8 ± 18.3 (6–90)	**<0.001**	0.726
- Maturity fears	37.5 ± 17.0 (10–86)	42.7 ± 16.8 (8–100)	0.064	0.302
Neurotensin (ng/ml)	0.8 ± 0.3 (0.1–1.8)	0.8 ± 0.5 (0.2–3.6)	0.835	0.034
Xenin (ng/ml)	0.9 ± 0.4 (0.3–2.4)	1.0 ± 0.5 (0.3–2.9)	0.131	0.247

Data are expressed as mean ± SD, the range is indicated in parentheses. Differences between the two groups were assessed using t-test. Significant p values are indicated in bold. Cohen's d is indicated as marker for the effect size. BMI, body mass index; EDI-2, Eating Disorder Inventory-2; GAD-7, Generalized Anxiety Disorder-7; PHQ-9, Patient Health Questionnaire-9; PSQ-20, Perceived Stress Questionnaire-20.

TABLE 2 | Demographic and socioeconomic characteristics, comorbidities and medication.

Parameter	Whole population (*n* = 160)	Women (*n* = 97)	Men (*n* = 63)	*p*	φ / *V*
Socioeconomic characteristics					
Living in a partnership	64 (40%)	41 (42.27%)	23 (36.51%)	0.467	−0.057
Level of education				0.246	0.184
University entrance diploma	35 (21.88%)	16 (16.49%)	19 (30.16%)		
Vocational diploma	8 (5%)	5 (5.15%)	3 (4.76%)		
Secondary education certificate	65 (40.63%)	44 (45.36%)	21 (33.33%)		
Basic school qualification	38 (23.75%)	22 (22.68%)	16 (25.4%)		
No school-leaving qualification	14 (8.75%)	10 (10.31%)	4 (6.35%)		
Currently employed	45 (28.13%)	26 (26.8%)	19 (30.16%)	0.645	0.036
Unemployment during past 5 years	71 (44.38%)	41 (42.27%)	30 (47.62%)	0.506	0.053
Comorbidities					
Bulimia Nervosa	2 (1.25%)	2 (2.06%)	0 (0%)	0.251	0.091
Binge-eating disorder	32 (20%)	27 (27.84%)	5 (7.94%)	**0.002**	0.243
Sleep-associated breathing disorder (women: *n* = 79; men: *n* = 56)	64 (47.41%)	28 (35.44%)	36 (64.29%)	**<0.001**	−0.258
Type 2 diabetes mellitus	52 (32.5%)	29 (29.9%)	23 (36.51%)	0.383	−0.069
Arterial hypertension	102 (63.75%)	52 (53.61%)	50 (79.37%)	**<0.001**	−0.262
Hypercholesterinemia	87 (54.38%)	45 (46.39%)	42 (66.6%)	**0.012**	−0.199
Hypertriglyceridemia	43 (26.88%)	20 (20.62%)	23 (36.51%)	**0.027**	−0.175
Hyperuricemia (women: *n* = 89; men: *n* = 61)	69 (46%)	38 (42.7%)	31 (50.82%)	0.327	−0.080
Fatty liver disease (women: *n* = 75; men: *n* = 43)	79 (66.95%)	46 (61.33%)	33 (76.74%)	0.087	−0.158
Medication					
Insulin	18 (11.25%)	7 (7.22%)	11 (17.46%)	**0.045**	−0.158
DPP-4 antagonists/GLP-1 analogs	10 (6.25%)	5 (5.15%)	5 (7.94%)	0.478	−0.056
Antidiabetics (other)	32 (20%)	18 (18.56%)	14 (22.22%)	0.571	−0.045
Proton-pump inhibitor	49 (30.63%)	27 (27.84%)	22 (34.92%)	0.342	−0.075
Non-steroidal anti-inflammatory drugs	50 (31.25%)	29 (29.90%)	21 (33.34%)	0.647	−0.036
Psychopharmacological treatment	59 (36.88%)	39 (40.21%)	20 (31.75%)	0.278	0.086
Neuroleptics	19 (11.88%)	12 (12.37%)	7 (11.11%)	0.810	0.019
SSRI/SNRI	39 (24.38%)	28 (28.87%)	11 (17.46%)	0.101	0.130
Tricyclic antidepressants	10 (6.25%)	7 (7.22%)	3 (4.76%)	0.531	0.050
Other antidepressants	9 (5.63%)	6 (6.19%)	3 (4.76%)	0.703	0.030
Tranquilizers, sedatives, hypnotics	2 (1.25%)	1 (1.03%)	1 (1.59%)	0.757	−0.024
Other psychopharmacological medication	10 (6.25%)	6 (6.19%)	4 (6.35%)	0.967	−0.003

Statistical analysis: Data are expressed as mean ± standard deviation or absolute number with percentage in parentheses. Differences between two groups were assessed using the chi-square test. Significant differences are displayed in bold. Phi-coefficient (φ) or Cramér's V (Level of Education) are indicated as markers for the effect size. BMI, body mass index; DPP-4, dipeptidyl peptidase-4 inhibitor; GLP-1, glucagon-like peptide-1; SNRI, serotonin-norepinephrine reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor.

for one man, whereas, EDI-2 data was lacking in one woman. Regarding ELISA analyses, one outlier for neurotensin and two outliers for xenin (all in women) were excluded from further statistical calculations. In the whole study population, the mean plasma neurotensin concentration was 0.8 ± 0.5 ng/ml (range: 0.1–3.6 ng/ml), mean plasma xenin was 0.9 ± 0.5 ng/ml (range: 0.3–2.9 ng/ml).

Psychometric parameters showed significant differences between gender groups (**Table 1**). Women displayed higher values for all psychometric parameters, namely perceived stress (+24%), anxiety (+37%), depressiveness (+32%), and eating disorder symptoms (+27%). Female and male participants did not show statistical differences concerning socioeconomic characteristics (**Table 2**). As shown in **Table 2**, female obese patients more often suffered from binge-eating disorder (BED) than male patients, while men showed significantly more somatic comorbidities. Insulin was a significantly more frequent component of medication in men (**Table 2**). However, testing these comorbidities and medication as potential confounders with help of multivariable linear regression indicated no significant influence, neither on neurotensin nor on xenin (**Supplementary Table 1**).

Patients with diabetes mellitus type 2 did not show differences in concentrations of neurotensin and xenin compared to those without diabetes (*t*-test; data not shown). Likewise, the peptide levels were similar in patients taking antidiabetic medication like insulin, DPP-4 antagonists/GLP-1 analogs or other antidiabetics, and in those without (*t*-test; data not shown).

After we excluded three women with polycystic ovary syndrome (PCOS), no significant difference of xenin concentrations was detectable ($p = 0.900$; $d = 0.018$).

Furthermore, there was no significant difference in plasma peptide levels between patients taking proton pump inhibitors (PPIs) or non-steroidal anti-inflammatory drugs (NSAIDs) and those not (*t*-test; data not shown). Fatty liver disease did not demonstrate a significant impact on neurotensin and xenin (*t*-test; data not shown). Also, when using linear regression for testing, we did not observe a significant predictive power of PPI's, NSAID's or of the diagnosis fatty liver disease, neither on neurotensin, nor xenin (**Supplementary Table 2**).

We also conducted multivariable linear regression analyses in order to estimate the influence of age and gender on xenin and neurotensin (xenin: $R^2_{cor} = 0.002$; $p = 0.319$; neurotensin: $R^2_{cor} = -0.012$; $p = 0.968$). Additionally, multivariable linear regression analyses for age and perceived stress, anxiety, depressiveness and eating disorder pathology, respectively, in women and men were calculated (**Supplementary Table 1**).

Sex-Specific Associations Between Neurotensin and BMI, Age, and Psychometric Parameters

In men but not in women, a significant weak negative correlation was observed between BMI and neurotensin plasma levels (**Table 3**). Strong positive correlations were observed between neurotensin and xenin in women and men (**Tables 3**, **4**).

TABLE 3 | Correlation of neurotensin with anthropometric, psychometric and endocrine parameters.

Parameter	Men (*n* = 63)		Women (*n* = 97)	
	r	*p*	*r*	*p*
Age (years)	0.113	0.378	−0.028	0.787
BMI (kg/m^2)	−0.265	**0.036**	−0.100	0.334
PSQ-20 total score	0.137	0.289	0.231	**0.023**
- Worries	−0.037	0.777	0.188	0.066
- Tension	0.145	0.262	0.274	**0.007**
- Joy	−0.142	0.271	−0.204	**0.046**
- Demands	0.208	0.105	0.113	0.273
GAD-7 total score	0.027	0.833	0.265	**0.009**
PHQ-9 total score	0.052	0.687	0.281	**0.006**
EDI-2 total score	−0.200	0.115	0.276	**0.007**
- Drive for thinness	−0.180	0.157	0.212	**0.039**
- Bulimia	−0.192	0.132	0.172	0.096
- Body dissatisfaction	−0.237	0.062	0.039	0.706
- Ineffectiveness	−0.065	0.613	0.229	**0.026**
- Perfectionism	0.046	0.719	0.336	**0.001**
- Interpersonal distrust	−0.117	0.360	0.201	0.051
- Interoceptive awareness	−0.064	0.620	0.252	**0.014**
- Maturity fears	−0.149	0.244	0.027	0.796
Xenin (ng/ml)	0.731	**<0.001**	0.788	**<0.001**

Correlations were assessed using Pearson's analysis. Significant correlations are indicated in bold. BMI, body mass index; EDI-2, Eating Disorder Inventory-2; GAD-7, Generalized Anxiety Disorder-7; PHQ-9, Patient Health Questionnaire-9; PSQ-20, Perceived Stress Questionnaire-20.

TABLE 4 | Correlation of xenin with anthropometric, psychometric and endocrine parameters.

Parameter	Men (*n* = 63)		Women (*n* = 97)	
	r	*p*	*r*	*p*
Age (years)	0.115	0.368	−0.041	0.696
BMI (kg/m^2)	−0.089	0.487	0.045	0.664
PSQ-20 total score	−0.040	0.760	0.254	**0.013**
- Worries	−0.146	0.257	0.205	**0.046**
- Tension	0.020	0.878	0.267	**0.009**
- Joy	0.042	0.745	−0.230	**0.025**
- Demands	0.031	0.812	0.158	0.127
GAD-7 total score	−0.088	0.495	0.257	**0.012**
PHQ-9 total score	−0.092	0.472	0.241	**0.019**
EDI-2 total score	−0.152	0.233	0.260	**0.011**
- Drive for thinness	0.021	0.868	0.274	**0.008**
- Bulimia	−0.142	0.265	0.096	0.359
- Body dissatisfaction	−0.168	0.189	0.177	0.088
- Ineffectiveness	−0.138	0.282	0.242	**0.019**
- Perfectionism	0.158	0.215	0.407	**<0.001**
-Interpersonal distrust	−0.169	0.185	0.140	0.178
- Interoceptive awareness	−0.133	0.298	0.167	0.107
- Maturity fears	−0.108	0.398	−0.076	0.468
Neurotensin (ng/ml)	0.731	**<0.001**	0.788	**<0.001**

Correlations were assessed using Pearson's analysis. Significant correlations are indicated in bold. BMI, body mass index; EDI-2, Eating Disorder Inventory-2; GAD-7, Generalized Anxiety Disorder-7; PHQ-9, Patient Health Questionnaire-9; PSQ-20, Perceived Stress Questionnaire-20.

In women, weak positive correlations between neurotensin and the total scores of the PSQ-20 (**Figure 1**), GAD-7 (**Figure 2**), PHQ-9 (**Figure 3**), and EDI-2 (**Figure 4**) were observed, while no such associations were observed in men (**Table 3**). However, reflecting weak positive correlations (**Table 3**), no significant association in multivariable linear regression for the independent variables of psychometrics (PSQ-20 total score; GAD-7; PHQ-9 and EDI-2 total score) and neurotensin was detectable in women ($R^2_{cor} = 0.052$; $p = 0.066$).

Sex-Specific Associations Between Xenin and BMI, Age, and Psychometrics

Weak positive associations of xenin and the total scores of all psychometric questionnaires were shown in obese women: PSQ-20 (**Figure 1**), GAD-7 (**Figure 2**), PHQ-9 (**Figure 3**), and EDI-2 (**Figure 4** and **Table 4**). Due to the weakness of correlations (**Table 4**), no significant association in multivariable linear regression between the independent variables of psychometrics (PSQ-20 total score; GAD-7; PHQ-9 and EDI-2 total score) and xenin was detectable in women ($R^2_{cor} = 0.036$; $p = 0.123$). In men, no significant correlations of the total and sub scores of applied psychometrics with xenin were detected (**Table 4**).

DISCUSSION

The aim of the present study was to examine potential associations between neurotensin and xenin in plasma

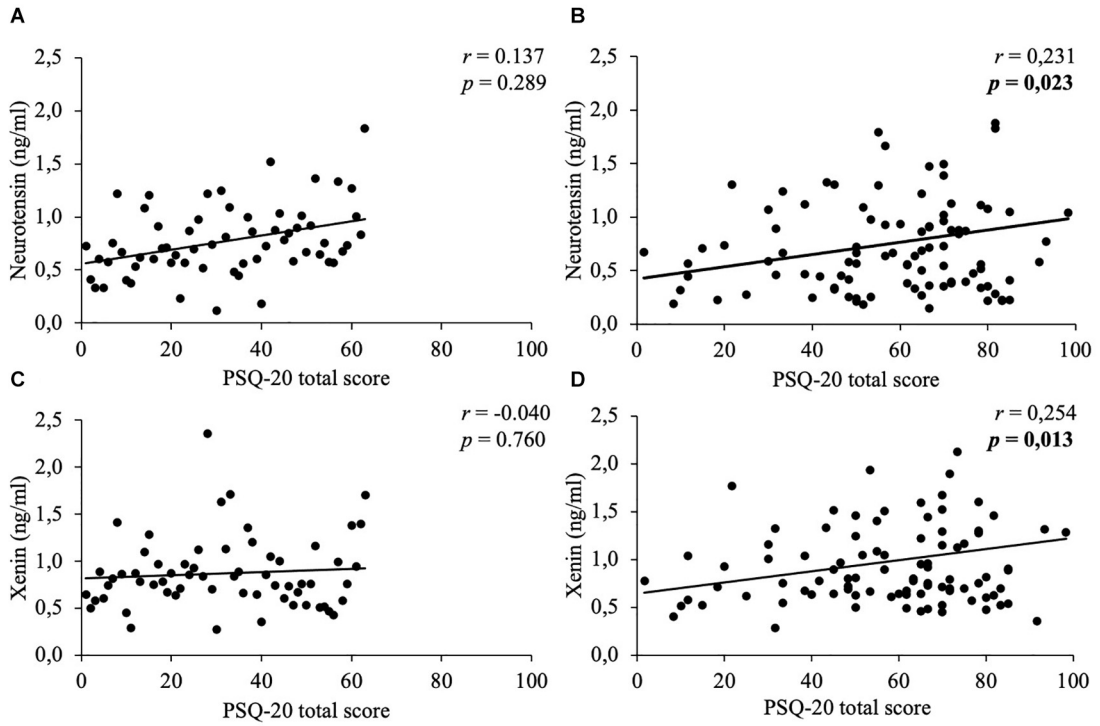

FIGURE 1 | Correlations between neurotensin and xenin with PSQ-20 total scores in men and women. Overall, in women neurotensin **(B)** and xenin **(D)** showed a positive correlation with PSQ-20 total score. However, for men no significant correlations with PSQ-20 total score were detected, neither for neurotensin **(A)** nor for xenin **(C)**. Correlations were calculated using Pearson's analysis. PSQ-20, Perceived Stress Questionnaire-20.

with psychometrically assessed perceived stress, anxiety, depressiveness and eating disorder symptoms in patients with obesity. Positive correlations of neurotensin and xenin with the above-mentioned psychometric scores were only detectable in obese women, whereas, no correlations were observed in obese men.

Mood and anxiety disorders are more prevalent in obese compared to normal weight subjects (Baumeister and Härter, 2007; Herpertz, 2015). A current study (n = 2,955) demonstrated increased adjusted odds ratios (OR) for obese participants of the German National Health Interview and Examination Survey – Mental Health Supplement at a twelve-months follow up for mood and anxiety disorders (Jacobi et al., 2004; Baumeister and Härter, 2007). Especially obese women suffer more often than obese men from mental comorbidities like anxiety and mood disorders and pathologic eating behavior (Gater et al., 1998; Preiss et al., 2013; McHenry et al., 2014; Herpertz, 2015; Tronieri et al., 2017). This observation is in line with our current data showing higher values of determined psychological characteristics in women.

Detected sex-differences might be additionally influenced by variations in obesity-related stigmatization between women and men. The stigmatization appears in all facets of everyday life, verbal and non-verbal, with direct consequences regarding for example eating habits (Faith et al., 2002; Schwartz et al., 2003; Puhl and Brownell, 2006). In previous literature, differences regarding stigmatization exist between obese women and men, whereby women feel more often affected (Puhl et al., 2008). Especially middle-aged and morbidly obese females report great psychological distress (Puhl et al., 2008). This current stage of research is in line with our finding of higher psychometric values in obese females. Moreover, the risk for the development of BED seems to be associated with a high level of perceived weight-related stigmatization (Almeida et al., 2011), which is reflected by our result of significantly more BED diagnoses among women. Potential influencing factors in this context could be the increased emotional awareness in addition to the cognitive skill of women to interpret facial expressions of emotions better than men, as shown in several studies (Hall and Matsumoto, 2004; Mankus et al., 2016). Higher values of our female obese patients in the EDI-2 subscale "interoceptive awareness" is a supportive indicator for this influence.

Studies on various animal models showed decreased central concentrations of neurotensin under conditions of obesity (Williams et al., 1991b; Beck et al., 1992). In the periphery, controversial data have been obtained: decreased peripheral concentrations of neurotensin were detected in morbidly obese patients (Weiss et al., 2001), while another study described a higher risk for high fat diet-induced obesity in mice with higher concentrations of peripheral pro-neurotensin (Li et al., 2016) possibly reflecting species differences. Decreased concentrations of xenin in plasma of obese children were observed, whereas, obese

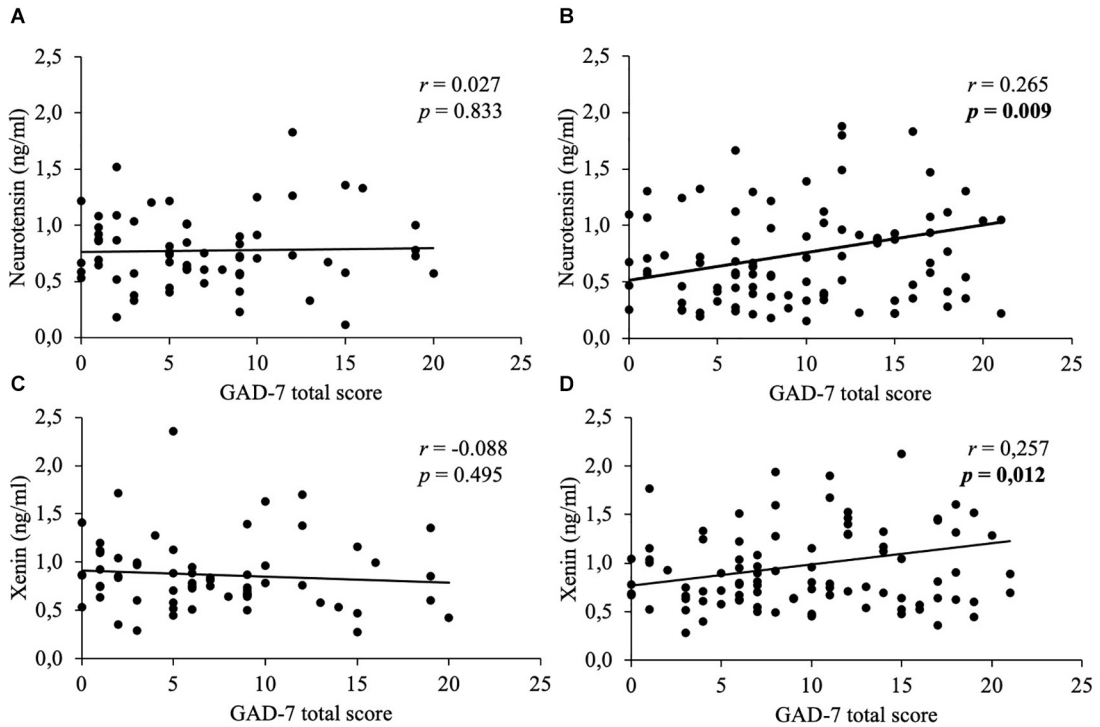

FIGURE 2 | Correlations between neurotensin and xenin with GAD-7 total scores in men and women. Overall, in women neurotensin **(B)** and xenin **(D)** showed a positive correlation with GAD-7 total score, while for men no significant correlations with GAD-7 total score were detected, neither for neurotensin **(A)** nor for xenin **(C)**. Correlations were calculated using Pearson's analysis. GAD-7, Generalized Anxiety Disorder-7.

adolescents showed increased serum levels (Mrozek et al., 2012; Arslan et al., 2014) pointing toward confounding factors such as comorbid psychiatric diseases influencing this association.

In the current study a negative association between neurotensin and BMI of obese men has been detected, which is in line with a previous study (Weiss et al., 2001), whereas, in other studies no difference in neurotensin levels between obese and lean subjects has been detected (Service et al., 1986; Auguet et al., 2018). However, these previous examinations bear several methodical limitations (small sample size) (Service et al., 1986), and methodical differences (gender distribution) (Weiss et al., 2001; Auguet et al., 2018). Thus, the comparability between results of these studies is limited. We assumed a positive association between psychometric parameters and BMI because of an increased number of comorbidities and elevated psychological strain with higher BMI. Consequently, the negative correlation might conceal higher plasma levels of neurotensin related to higher scores of questionnaires in morbidly obese men. However, one has to note that only an obese spectrum of body weight was assessed in the present study.

In light of the receptor distribution of NTS_1 in certain brain areas, a role of neurotensin and xenin in the regulation of stress and emotions can be assumed since NTS_1 is highly expressed in the amygdala and limbic system (Alexander and Leeman, 1998; Fassio et al., 2000). Regarding neurotensin, both anxiogenic and anxiolytic effects have been demonstrated in rats after injection into the bed nucleus of stria terminalis (BNST; anxiogenic) or ventral pallidum (anxiolytic) (Ollmann et al., 2015; Normandeau et al., 2018; Li et al., 2020) indicating a site-dependent effect of the peptide. Mean concentrations of neurotensin and xenin did not differ between female and male obese patients in our study, while only women showed positive correlations of both peptides with the examined psychological parameters giving rise to a sex-dependent association. A sex-specific regulation of neurotensin has been suggested previously. The peptide hormone is considerably higher concentrated in the anteroventral periventricular nucleus in female rats, influences the release of gonadotropin-releasing-hormone and in turn is itself affected by menstrual cycle and estrogens (Alexander et al., 1991; Herbison and Theodosis, 1991, 1992; Alexander, 1993; Dungan Lemko et al., 2010). As a consequence, also dysregulated levels of reproductive hormones in morbidly obese men may have an influence on central neurotensin concentrations and furthermore on the sex-dependent associations with psychometrics (Hammoud et al., 2008). Xenin is less investigated regarding potential sex-dependent differences compared to neurotensin. A case-control study showed an increase of serum xenin in women with PCOS versus women without PCOS (Norman et al., 2007; Azziz et al., 2016; Guclu et al., 2019). However, co-morbid PCOS of our female obese inpatients (three women) did not contribute to divergences of the examined associations.

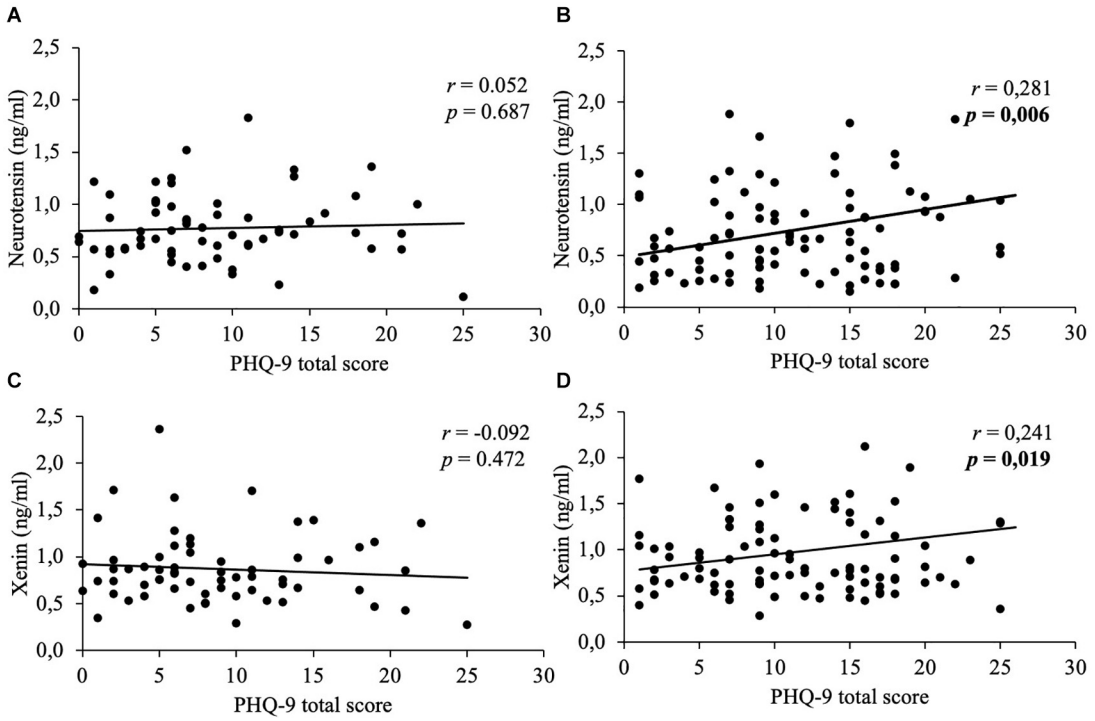

FIGURE 3 | Correlations between neurotensin and xenin with PHQ-9 total scores in men and women. Overall, in women neurotensin **(B)** and xenin **(D)** showed a positive correlation with PHQ-9 total score. However, for men no significant correlations with PHQ-9 total score were detected, neither for neurotensin **(A)** nor for xenin **(C)**. Correlations were calculated using Pearson's analysis. PHQ-9, Patient Health Questionnaire-9.

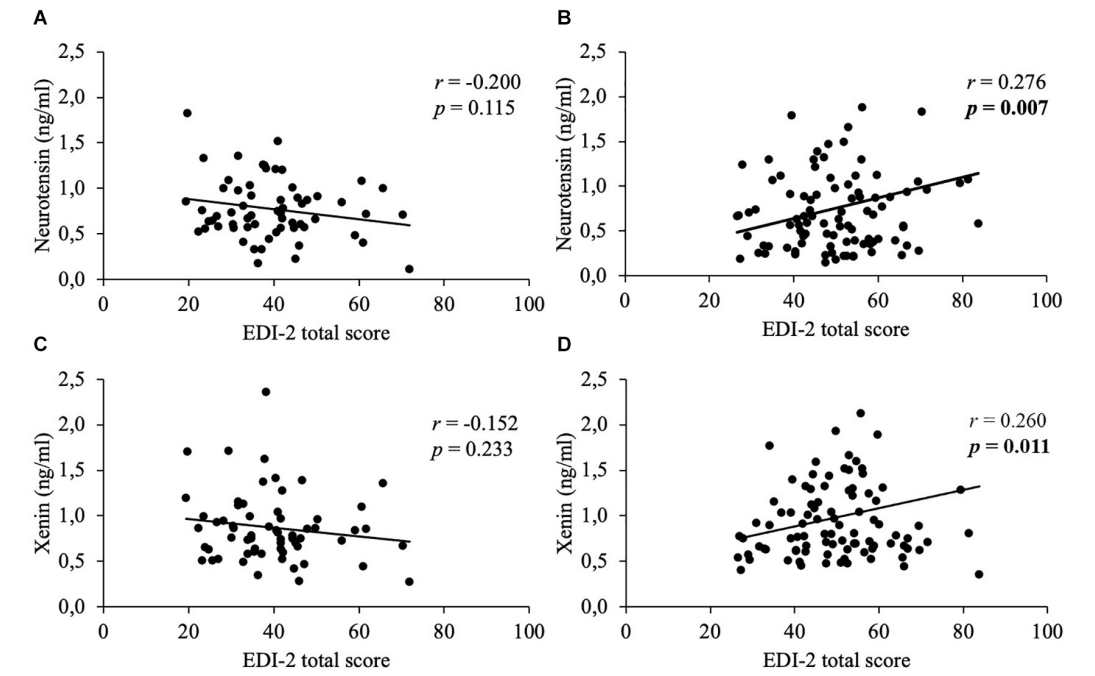

FIGURE 4 | Correlations between neurotensin and xenin with EDI-2 total scores in men and women. Overall, in women neurotensin **(B)** and xenin **(D)** showed a positive correlation with EDI-2 total score, while for men no significant correlations with EDI-2 total score were detected, neither for neurotensin **(A)** nor for xenin **(C)**. Correlations were calculated using Pearson's analysis. EDI-2, Eating Disorder Inventory-2.

Therefore, sex hormones should be additionally analyzed in future research.

Neurotensin and stress have been closely linked. Early on it was shown that icv injection of neurotensin stimulates the secretion of adrenocorticotropic hormone (ACTH) and subsequently the adrenal glands in rodents (Gudelsky et al., 1989). Recently, it was shown that chronic stress induces central neurotensin release in rats (Normandeau et al., 2018). Orexigenic signaling through increased HPA axis hormones is a further stimulus for obesity (Strack et al., 1995; Chrousos, 2009) and might counteract the anorexigenic effect of neurotensin in CNS. This may indicate that higher neurotensin levels contribute to the development and/or maintenance of perceived stress and anxiety. For xenin, we found positive correlations with perceived stress and anxiety in women with obesity. In a previous study, a linear correlation between peripherally measured and centrally xenin levels gave rise to a transport across the blood-brain barrier (BBB) (van de Sande-Lee et al., 2013). Accordingly, given the current controversial state of research, elevated xenin levels in the CNS of obese women together with agonistic effects of the peptide at NTS_1 may be an indicator for anxiogenic and stress-inducing consequences (Steele et al., 2017; Normandeau et al., 2018; Li et al., 2020). However, clarification of the signaling pathways is necessary to obtain a more detailed picture.

In the current study, depressiveness was positively correlated with neurotensin and xenin in obese women only. In this context, it is to note that female obese patients more often show a comorbid major depression compared to men (Herpertz, 2015). Moreover, several items of the PHQ-9, such as "dysregulated appetite" and "low self-esteem," overlap with facets of other psychometric questionnaires (e.g., EDI-2) (Garner, 1991; Thiel et al., 1997; Löwe et al., 2002). This might suggest that correlations refer at least partially to similar constructs. In addition, mood disorders are common comorbidities of eating disorders, in adolescence and also over the whole lifespan (Casper, 1998; Swanson et al., 2011). A recent study reported a potential association between plasma neurotensin and the number of lifetime suicide attempts in depressive patients, with the result of a strong positive correlation (Kim et al., 2019) further suggesting an involvement of neurotensin in the pathogenesis of depression.

Similarly, neurotensin and xenin were correlated with parameters of disordered eating in obese women only. This might be related to higher prevalence rates of eating disorders in women, especially for anorexia nervosa (AN) and for bulimia nervosa (BN), with up to tenfold higher prevalence in women (Arbeitsgemeinschaft Wissenschaftlicher Medizinischer Fachgesellschaften, 2018). BED was more prevalent in our female participants, which might be contributing to the lacking correlation in men. Furthermore, an association of sex hormones with disordered eating behavior and obesity is notable (Hirschberg, 2012). Testosterone levels are elevated in obese women following increased chronic stress and hyperinsulinemia, which promotes binge eating and abdominal fat distribution (Torres and Nowson, 2007; Hirschberg, 2009, 2012). Moreover, the HPA axis is overstimulated through endocrine activity of the abdominal fat (Torres and Nowson, 2007; Pasquali et al., 2008; Hirschberg, 2009, 2012). In obese men, estradiol concentrations are elevated and the quantity of abdominal fat leads to lower testosterone concentrations (Hammoud et al., 2008; Pasquali et al., 2008; Hirschberg, 2012). Data concerning the relationship of xenin with depressiveness or disordered eating behavior are lacking, but due to same receptor and the high sequence homology, similar explanations as for neurotensin may be assumed (Feurle, 1998; Maes et al., 2001; Kim and Mizuno, 2010b; Li et al., 2020). Thus, future studies should focus on xenin.

Several limitations of the study should be kept in mind. First, we did not assess the menstrual status, and the intake of estrogen-containing medication was not considered as an exclusion criterion. Second, due to the naturalistic design no healthy control group was employed in our study. We therefore were not able to compare our findings in obese subjects with normal weight individuals without mental disorders under study conditions. However, the naturalistic design is also a strength of this examination because it reproduces real world conditions during psychosomatic inpatient treatment. Third, the cross-sectional study design observes associations, no cause-effect relationships. Hence, besides experimental studies featuring healthy control groups, future longitudinal studies are required assessing neurotensin and xenin levels in the course of psychopathology-improving therapies. Lastly, heterogeneous comorbidities are potentially representing confounding factors and therefore might contribute – besides the relatively small sample size – to the observed weak associations. Thus, further studies with bigger sample size or with more stratified study collectives are needed.

Based on previous literature, increased xenin levels following treatment with PPI's as well as decreased plasma concentrations of neurotensin in women with fatty liver disease and obesity were described (Stoschus et al., 1998; Auguet et al., 2018). Previous studies indicated that gastrointestinal (initiation of gut motility and inhibition of gastric acid production) and central effects of neurotensin depend on prostaglandin (Mason et al., 1982; Katsoulis and Conlon, 1988), why we investigated a possible influence of the intake of NSAID's on peptide levels. Until know, interactions between NSAID's and xenin were not assessed, but imaginable (Stoschus et al., 1998; Kapraali et al., 1999). However, in our study NSAID's did not affect peripheral levels of neurotensin or xenin.

Taken together, we observed positive correlations of neurotensin and xenin with stress, anxiety, depressiveness and eating disorder symptoms in obese women, whereas, associations were absent in male patients with obesity indicating a sex-specific association. Whether this association represents a causal relationship should be further investigated. If so, it would be interesting to investigate whether inhibition of these peptides could exert a beneficial effect on these psychometric parameters. Lastly, due to the sequence homology between neurotensin and xenin along with the – at least in part – similar activation of the NTS_1 it will be interesting to investigate whether the peptides are be able to compensate each other.

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 Institutional Ethics Committee of the Charité – Universitätmedizin Berlin (protocol number: EA1/130/16). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EW and SS collected the samples. EW analyzed the data and wrote the first draft of the manuscript. TH and AS designed the study. TH, MR, and AS gave critical input throughout the work. AS analyzed the data. All authors finalized and agreed on 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/fnbeh.2021.629729/full#supplementary-material

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Toward Understanding the Sex Differences in the Biological Mechanism of Social Stress in Mouse Models

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Significant sex differences in terms of prevalence, symptomatic profiles, severity, and comorbidities of psychiatric disorders are quite common. Women have been shown to be more vulnerable to stress and are nearly twice as likely as men to develop stress-related disorders such as depression and anxiety. Therefore, understanding sex differences with respect to the neurobiological mechanisms underlying stress-related disorders is important for developing more efficient pharmacological interventions for women. However, most preclinical studies on stress-related disorders have focused heavily on male rodents. Here, recent developments in the study of repeated social defeat stress models in female mice are summarized. Our findings suggest that a variety of factors need to be considered when employing this model.

Keywords: social stress, female, mouse, sex difference, repeated social defeat stress model (RSDS)

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Chronic subordination and repeated defeat experiences induce prolonged stress responses and cause several stress-related behaviors, such as exaggerated anxiety, social avoidance, anhedonia, and behavioral despair (1–4). Thus, the repeated social defeat stress (RSDS) model has been widely employed for studying neurobiological mechanisms underlying stress-related disorders. RSDS has long-term effects on stress-related behaviors, which are reversed by chronic—but not acute—treatment with antidepressants (5). Importantly, the RSDS model allows researchers to study the individual differences in stress susceptibility, and about half of the socially defeated animals show depressive-like symptoms (6). Therefore, although several models of depression exist (i.e., forced swim test, tail suspension test, chronic mild stress, uncontrollable stress, and olfactory bulbectomy), the RSDS model is considered to have high etiological, predictive, and face validity (7). However, RSDS studies have traditionally been restricted to males because this model relies on intermale territorial aggression. Female mice and rats are considered to have low levels of aggression, except when they are mothers (maternal aggression), and male-to-female aggression is rare in these species. Recently, efforts have been made to establish female RSDS models in mice because they are considered malleable to genetic modification and other neurobiological techniques (**Table 1**).

One approach involved artificially inducing female-directed aggression by manipulating the activity of the hypothalamic attack area of aggressor animals. Following the chemogenetic activation of estrogen receptor alpha (ERα)-positive neurons in the ventrolateral subdivision of the ventromedial hypothalamus (VMHvl), the male aggressor mice exhibited constant and intense aggressive behavior toward females, which was comparable to inter-male aggression (8). Defeated females in this model exhibited social avoidance and increased anxiety-like behaviors (8, 9).

TABLE 1 | Female social defeat stress mouse models.

	Male to female					Female to female		Witness
Manipulation	Gq-DREADD activation of the VMHvl of aggressor male by CNO i.p. injection (2)			Application of male urine to the base of the tail and the vaginal orifice of test female (6)	Simultaneous intoduction of male and female C57BL/6J into aggressor's homecage (7)	Pair housed female aggressor with a male (either intact or castrated) (8)		Witnessing social defeat in other male C57BL/6 (9)
Subject	C57BL/6J female			C57BL/6J female	C57BL/6J female	C57BL/6J female		C57BL/6 female
Age	8 weeks			3–4 months	8 weeks	12 weeks		10 weeks
Aggressor	ERα-Cre male mice with hM3D expression in the VMHvl			CD1 male	CD1 male	CFW female		CD1 male attack C57BL/6 male
Defeat duration	10 min, 10 day	5 min, 10 day		5 min, 10 day	5 min, 10 days	Acute: 5 min, 1day	Chronic: 5 min, 10day	10 min, 10 days
Attacked days	ave 9.6 days			median 5 days	64.33% of interaction			0
Housing condition	**Sensory contact**	**Individual housing**	**Paired with another female**	**Sensory contact**	**Sensory contact**	**Individual housing**	**Sensory contact**	**Sensory contact**
Social interaction test	↓	↓	↓	↓	↓	-	↓	↓
	S<R=C	S<R=C	S<R=C	S<R=C	S<R=C	(direct interaction)		
Rate of susceptible	10–19%	50%	50%	58%	About 63%	na	na	na
Effect of estrous cycle	-	-	-	-	-	na	na	na
Anxiety-like behavior	na	-*	↑	↑	↑	na	-	(↑)
			S=R>C	S=R>C	S>R=C			
Anhedonia	na	na	na	↑	↑	na	na	↑
				S>R=C	S>R=C			
Body weight	na	-	↓	na	na	na	-	↓
			S<R=C					
Corticosterone	na	na	na	↑	↑	↑	↑	↑
					S>R=C			

**No effect of RSDS due to a reduction of body weight and an increased anxiety-like behavior in control animals by individual housing.*

Anhedonia: reduction of sucrose preference, Sensory contact: Housed with an aggressor male via perforated divider. Susceptible and resilient were defined by social interaction behavior in the social interaction test. S, susceptible; R, resilient; C, control. Effect of estrus cycle indicates its effect on social interaction behavior.

↑, increase; ↓, decrease; -, no effect; na, not tested.

Importantly, consistent with the findings in males (6), large individual differences in social avoidance behavior were observed, and only the females that showed social avoidance—and were thus considered stress-susceptible—exhibited a reduction in body weight and increased expression of proinflammatory cytokines (8). Therefore, this model enables the investigation of the mechanisms underlying stress susceptibility and resilience in both males and females. Similarly, surgical lesions in the mediobasal hypothalamus of female rats induced aggressive behavior toward female intruders (10). However, this inter-female RSDS had a relatively mild effect on stress responses in defeated female rats, and they were more vulnerable to social instability stress, wherein social isolation and crowding stress were combined (10).

Another method uses male pheromones to induce male-to-female aggression. In one model, male urine was applied on the body of target female mice (11). Although the level of aggressive behavior toward urine-applied females was lower than that observed in intermale aggression, the application of urine induced similar behavioral changes in both sexes, including social avoidance and increased anxiety-like behaviors. Another method involved placing both male and female intruders simultaneously inside the territory of aggressor male mice, inducing non-specific aggressive behavior toward intruders of both sexes (12). Again, while female intruders in this model experienced fewer attacks than males, both sexes showed similar significant social avoidance, increased anxiety-like behaviors, and elevated blood corticosterone levels, particularly in susceptible animals.

More researchers are trying to develop more ethologically-natural models without any artificial intervention. For example, a recent study showed that a subpopulation of female mice (about 65%) exhibited aggressive behaviors toward female as rival aggression, which was comparable to those of males (13). When a female mouse was housed with a male mouse, even a castrated one, the resident females showed rival aggression toward the intruding female. RSDS with this interfemale aggression increased corticosterone levels, reduced social interaction and social hyperthermia, and disrupted nest building. In contrast, these females did not undergo changes in body weight or anxiety-like behaviors (13). Furthermore, witnessing defeat in other animals has been shown to induce depression-like behaviors, increase corticosterone, and reduce body weight in both male and female mice and rats (14, 15).

Using these models, RSDS has been shown to activate the immune system in a similar manner in male and female mice, including splenomegaly, increased myelopoiesis, and accumulation of monocytes in the spleen and brain (9). In addition, stress-susceptible females showed higher interleukin 6 (IL-6) levels compared with resilient and control female mice, consistent with findings in male mice (8). However, other cytokine responses differed between sexes. For example, female—but not male—mice exhibited significantly elevated levels of proinflammatory cytokines, such as IL-1α, IL-1β, IL-12, and TNF-α (in addition to IL-6), following the first defeat, indicating a broader inflammatory profile in females following a single defeat episode (16). In addition, gene expression profiles in the prefrontal cortex following RSDS differed between the sexes, with males showing approximately twice as many differentially expressed genes in response to RSDS compared with females (16). Thus, although the main biological pathway for social stress seems to be shared between males and females, there are some important distinctions between the sexes. One study using an RSDS mouse model showed that a long non-coding RNA whose expression is downregulated specifically in women with depression has antidepressant effects in female—but not male—mice (17). Therefore, the female RSDS model will provide important insights into sex differences in the mechanism of stress susceptibility, which will lead to the development of more efficient pharmacological interventions for stress-related disorders in women.

However, there are some factors that need to be considered when using the female RSDS model. It is likely that housing conditions have different effects on males and females. In males, sensory exposure to a dominant male aggressor over a perforated divider after physical defeat stress has been shown to enhance susceptibility; however, in females, this sensory exposure to an aggressor male induced a stress-resilient phenotype (8). However, social isolation imposed via individual housing strongly induced stress in female mice, but not in male mice, and even control females (without RSDS) in individual housing showed higher anxiety-like behaviors compared with group-housed females (8). Thus, there are important differences with respect to the effects of various social stresses or *stressors* (e.g., social defeat, social instability, sensory contact, and social isolation) on the sexes. In addition, the effects may also vary depending on whether the female is attacked by males or females. Inter-female aggression seems to have a milder effect on female behavior and physiology than male-to-female aggression. These facts make it difficult to directly compare stress susceptibility between males and females. To study the molecular and cellular mechanisms underlying social stress in female mice, appropriate test conditions that induce high levels of stress in females must be explored. Another important factor to be considered in the female model is the estrous cycle. However, in female RSDS models, the estrous cycle did not affect social avoidance or anxiety-like behaviors (**Table 1**). In addition, while prolonged stress causes the disruption of the estrous cycle in humans, disruption of the estrous cycle was not observed in the inter-female RSDS model in mice (13), and this aspect must therefore be examined in other models.

Although this is just the beginning of the employment of a female RSDS model to study the mechanisms underlying social stress and stress susceptibility in female mice, additional studies utilizing these models are expected to uncover important targets for the treatment of stress-related disorders in women.

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 authors.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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The Role of the Kappa Opioid System in Comorbid Pain and Psychiatric Disorders: Function and Implications

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Both pain and psychiatric disorders, such as anxiety and depression, significantly impact quality of life for the sufferer. The two also share a strong pathological link: chronic pain-induced negative affect drives vulnerability to psychiatric disorders, while patients with comorbid psychiatric disorders tend to experience exacerbated pain. However, the mechanisms responsible for the comorbidity of pain and psychiatric disorders remain unclear. It is well established that the kappa opioid system contributes to depressive and dysphoric states. Emerging studies of chronic pain have revealed the role and mechanisms of the kappa opioid system in pain processing and, in particular, in the associated pathological alteration of affection. Here, we discuss the key findings and summarize compounds acting on the kappa opioid system that are potential candidates for therapeutic strategies against comorbid pain and psychiatric disorders.

Keywords: kappa opioid system, pain-induced negative affect, cormorbid pain and psychiatric disorders, KOR agonists, KOR antagonists

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INTRODUCTION

Untreated negative affect induced by chronic pain largely drives vulnerability to mood disorders. Epidemiological evidence suggests that the prevalence of depression ranges from 30 to 80% in different pain etiologies (Bair et al., 2003; Howe and Sullivan, 2014), and chronic pain is one of the chief complaints in 65% of patients with treatment-resistant depression (Bair et al., 2003). Moreover, clinical studies have shown that comorbid chronic pain and depression mutually promote disease severity: such patients exhibit a poorer prognosis than those with only one disorder (Scherrer et al., 2016; Sullivan, 2016). Uncovering the mechanisms underlying comorbidity is therefore essential for proper treatment of these patients.

Owing to the complex pathogenesis of comorbid pain and psychiatric disorders, no existing animal model can mimic all of the relevant aspects. However, in recent years, scientists have begun to address the links between the two by dividing pain into sensory and affective dimensions and using behavioral paradigms to analyze affective states in animal models of chronic pain. The results have shown that many types of chronic pain induce aversion phenotypes in animals, including decreased motivation in goal-directed behaviors, conditioned place aversion, longer immobility times in the forced swim test, and decreased time in the light compartment during the light/dark test (Leite-Almeida et al., 2015). These behavioral observations in animals may reflect some of the

psychiatric symptoms observed in patients. Thus, uncovering the mechanisms underlying pain-induced aversive states in animals may be the key to understanding the comorbid relationship between chronic pain and mood disorders.

Opioids are the most effective prescription for relieving chronic pain. The classic opioid receptors, the mu opioid receptor (MOR), the delta opioid receptor (DOR), and the kappa opioid receptor (KOR), all belong to the class A (rhodopsin-like) γ-subfamily of seven-transmembrane G protein-coupled receptors (GPCRs), which forms the largest family of targets for current therapeutics. A complete GPCR system involves ligands, a receptor, and transducers. Upon binding to extracellular ligands, GPCRs often undergo a conformational change that causes GDP to be exchanged for GTP bound to Gα, leading to dissociation of Gα and the Gβγ dimer. Both the activated GTP-bound Gα and the Gβγ dimer can transduce signals via second messengers or transducers such as cAMP, inositol trisphosphate (IP_3), and diacylglycerol (DAG). After agonist activation, GPCRs are phosphorylated by G protein-coupled receptor kinases (GRKs), which recruit arrestin to the original G protein-binding sites. This process makes GPCRs lose the ability to respond to ligand binding, which is referred to as "receptor desensitization." Long-term use of opioids has been shown to cause addiction and increase the risk of depression (Crofford, 2010; Salas et al., 2017), which may be mediated by the desensitization of opioid receptors. These inherent adverse effects limit the clinical application of opioids. The classic theory of addiction hypothesizes that all addictive drugs enhance dopamine (DA) transmission in the reward circuitry. Interestingly, unlike the excitatory effects of MORs and DORs, KORs normally inhibit neuronal activity and neurotransmission (Tejeda et al., 2013, 2017). Systemic activation of KORs elicits analgesia similar to that induced by MOR activation but with fewer incidences of euphoria and reinforcement. Studies have demonstrated that MORs and KORs have opposite effects on the regulation of motivational processes (Spanagel et al., 1992). These features make KOR a promising drug target to develop non-addictive analgesics.

However, kappa opioid analgesics produce dysphoric effects and psychotomimesis in humans (Walsh et al., 2001; Chartoff and Mavrikaki, 2015) and elicit place aversion and depressive-like affective behaviors in rodents (Chavkin and Koob, 2016; Darcq and Kieffer, 2018). In fact, among the three classical opioid receptor systems, KORs in concert with their primary endogenous ligand, dynorphin, are most heavily implicated in aversion and psychiatric disorders such as depression and anxiety. Increased dynorphin release and KOR expression have been observed in suicidal individuals and preclinical models of neuropsychiatric disorders (Peckys and Hurd, 2001). KOR antagonists are capable of overcoming the pro-depression and anxiogenic effects of chronic or acute stressors (Carr et al., 2010; Browne et al., 2018), and a number of KOR antagonists are already in clinical trials for the treatment of psychiatric disorders (Lowe et al., 2014; Buda et al., 2015; Chavkin and Martinez, 2015). At the same time, KOR expression and function are significantly altered in various chronic pain models, such as peripheral nerve injury (Liu et al., 2019) and chronic constriction injury (Wawrzczak-Bargiela et al., 2020). However, whether the dynorphin/KOR (Dyn/KOR) system is directly involved in pain and psychiatric disorders remains unclear. Considering the opposing effects of KOR activation on chronic pain and depression, how to properly harness KOR agonists as analgesics while avoiding their side effects still needs to be determined. Here, we discuss the function of the Dyn/KOR system and its clinical implications and summarize promising candidates for the treatment of comorbid pain and psychiatric disorders.

KOR EXPRESSION AND SIGNALING

The Dyn/KOR system is distributed throughout different brain regions, with KORs expressed in various types of mood-related neurons including serotonergic neurons (Land et al., 2009), corticotropin-releasing factor (CRF) neurons (Marchant et al., 2007), and DA neurons (Liu et al., 2019; **Figure 1**). It is widely accepted that KOR activation produces negative affect, both in human beings and in rodents (Martinez et al., 2019). Moreover, ablation of KORs from DA neurons or basolateral amygdala (BLA) glutamatergic terminals in the medial prefrontal cortex produces an anxiolytic phenotype (Margolis et al., 2006; Lowery-Gionta et al., 2018), suggesting that the KOR system is critical for the expression of negative affect. Interestingly, besides the regions mentioned above, high levels of the precursor prodynorphin are detected in the periaqueductal gray, the striatum, and the bed nucleus of the stria terminalis (BNST) (Marchant et al., 2007). Given that elements of the Dyn/KOR system are present in the main circuitry involved in both pain processing and affective/motivational systems, it seems likely that the Dyn/KOR system contributes to the aversive nature of chronic pain. At the primary afferent level, KOR is expressed in a transcriptionally distinct subset of peptidergic afferents that strongly express the genes encoding calcitonin gene-related peptide (CGRP) and substance P, and in two populations of low-threshold mechanoreceptors; however, there are very low levels of KORs in cool-sensing neurons and proprioceptors (Snyder et al., 2018).

KORs are located both presynaptically and postsynaptically and play different roles. For example, in the dorsal raphe nucleus (DRN), acute KOR activation inhibits serotonergic neuronal excitability through presynaptic inhibition of excitatory synaptic transmission and postsynaptic activation of ion channels (Lemos et al., 2012). Within the BNST, KORs provide inhibitory control over presynaptic GABAergic signaling (Li et al., 2012; Hwa et al., 2020). Generally, presynaptic KORs modulate monoaminergic and glutamatergic neurotransmitter release whereas postsynaptic KORs hyperpolarize the cell membrane and inhibit neuronal excitation.

Like other GPCRs, most KOR signaling can be divided into two types: classical KOR signal-transduction pathways and KOR-induced arrestin-p38 mitogen-activated protein kinase (arrestin-MAPK) cascades (**Figure 2**). In the first signaling pathway, Gβγ released from the Gα subunit binds directly to ion channels and plays an inhibitory role by activating G protein-gated inwardly rectifying potassium channels ($K_{ir}3$s) or blocking voltage-gated Ca^{2+} channels (Rusin et al., 1997; Sadja et al., 2003). Meanwhile,

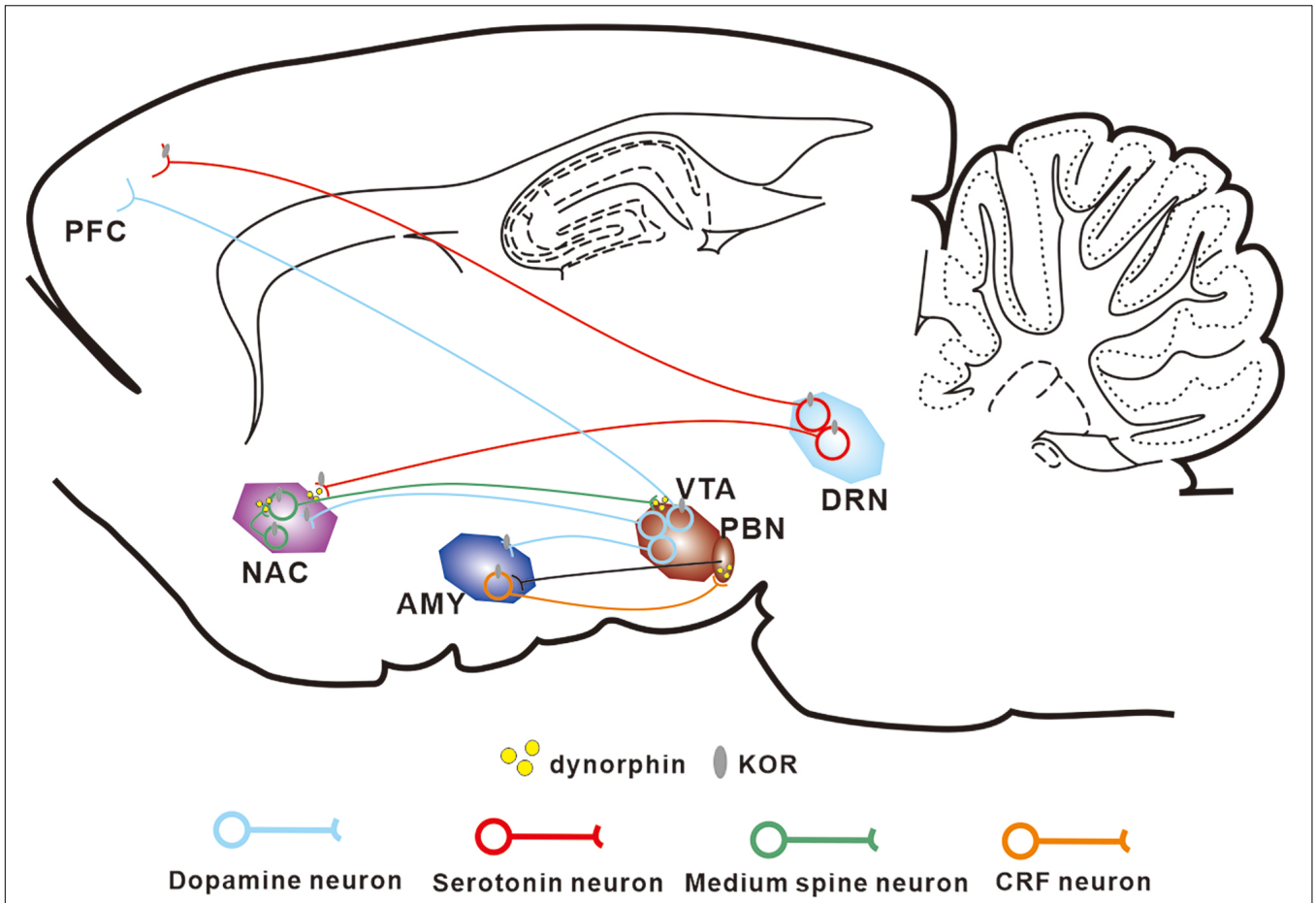

FIGURE 1 | Localization of the Dyn/KOR system within the major circuits involved in pain-induced aversion. KORs are found in major neuronal subtypes in a number of brain areas thought to be involved in pain-induced aversion. KORs modulate the interactions between these circuits through presynaptic and postsynaptic mechanisms. PFC, prefrontal cortex; NAC, nucleus accumbens; AMY, amygdala; VTA, ventral tegmental area; DRN, dorsal raphe nucleus.

Gα decreases cAMP and causes ERK phosphorylation. These inhibitory effects have been demonstrated in various cell types ranging from hippocampal granule cells to spinal cord motor neurons, resulting in decreased transmission of nociceptive stimuli at multiple levels of the pain pathways and a profound reduction in the perception of pain. However, several studies have reported excitatory effects of KOR activation, particularly with chronic KOR agonist treatment. This suggests that under some circumstances KORs can activate stimulatory G proteins and upregulate adenylyl cyclase, thus contributing to heterologous tolerance of opioids and physical dependence (Raynor et al., 1994; Avidor-Reiss et al., 1997).

In the second signaling pathway, KORs recruit β-arrestin scaffolding proteins after agonist-induced GRK3 phosphorylation of the C-terminal intracellular domain. It has been reported that phosphorylation at KOR serine 369 in rodents (Land et al., 2009) or serine 358 in humans (Li et al., 2002) leads to KOR desensitization and internalization. The recruited arrestin mediates p38 MAPK and ERK1/2 phosphorylation (Bruchas et al., 2008; McLennan et al., 2008) and has been observed to occur following behavioral stress. The β-arrestin signaling pathway has been demonstrated to contribute to KOR-induced aversion, dysphoria, and sedation, but not to KOR-induced analgesia. Thus, functionally selective KOR agonists, termed "biased agonists," may be able to selectively avoid p38 MAPK activation and hold promise for pain relief without side effects (as detailed in the section "Drug Candidates and Future Directions," below). Possible substrates of the KOR-activated p38 MAPK pathway include ion channels (Nav1.8, Kir3.1), serotonin transporters (SERT), and transcription factors (zif268, elF4B) (Hudmon et al., 2008; Steiner et al., 2008; Guan et al., 2010; Ruan et al., 2010). Regulation of these effectors may explain the characteristics of the behavioral responses seen with KOR-induced p38 activation. Arrestin-dependent KOR-induced ERK1/2 phosphorylation occurs 2 h after agonist treatment and is known as late-phase ERK1/2 phosphorylation; early phase ERK1/2 activation occurs 5–15 min after agonist treatment and depends on the Gβγ subunit. Potential effects of KOR-induced early phase ERK1/2 activation include increases in AMPA receptor cell surface expression, growth of dendritic spines, and regulation of CREB activation. Interestingly, KORs can

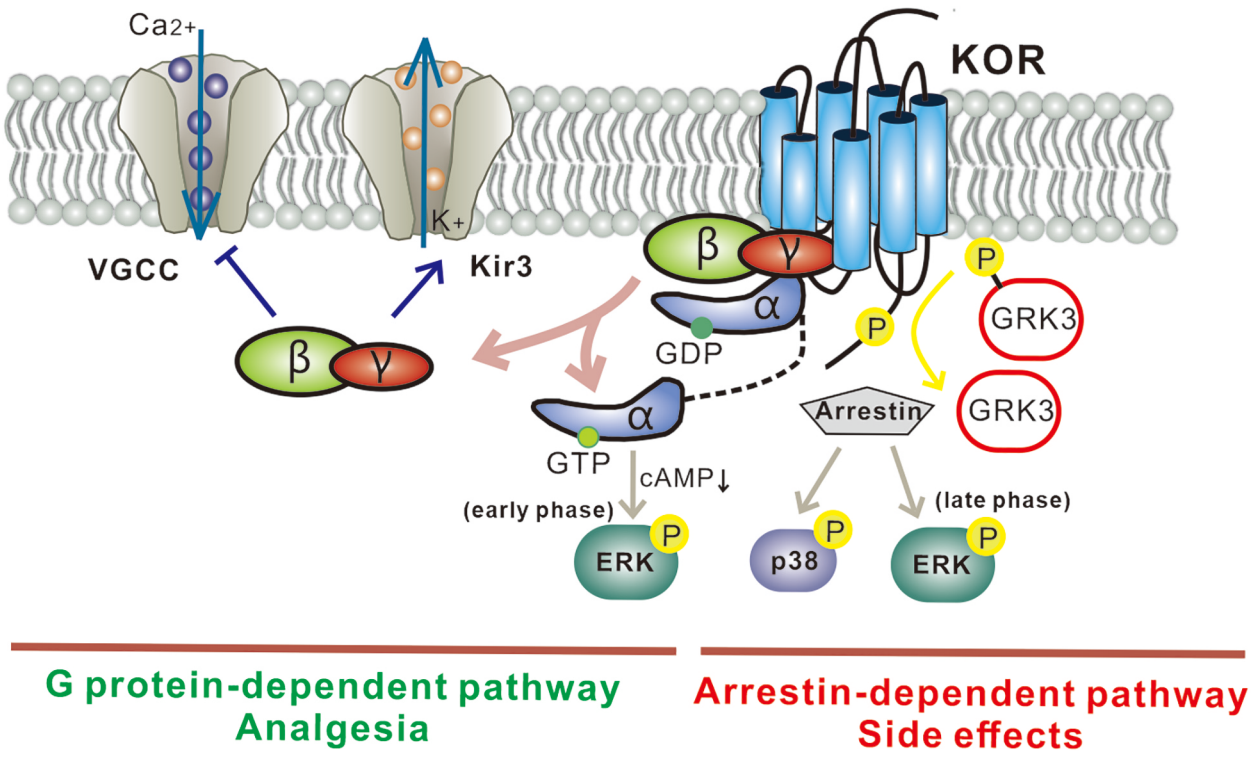

FIGURE 2 | Different downstream KOR signaling pathways mediate distinct functions. The G protein-mediated KOR signaling pathway plays an inhibitory role by activating G protein-gated inwardly rectifying potassium channels ($K_{ir}3$s) and blocking voltage-gated Ca^{2+} channels. Gα mediates a decrease in cAMP via Gαi and causes ERK phosphorylation (early phase: 5–15 min after agonist treatment). This classic pathway mediates the analgesia induced by KOR ligands. Separately, KOR activation also recruits arrestin and leads to the activation of several kinase cascades including ERK (late phase: 2 h after agonist treatment) and p38, which mediate the adverse effects induced by KOR ligands. Biased KOR agonism of the G protein-dependent pathway should lead to effective analgesia with fewer side effects. Arrows: activation steps, T lines: inhibition of function. Kir3, G protein-gated inwardly rectifying potassium channel; VGCC, voltage-gated Ca^{2+} channel; P, phosphorylation; cAMP, cyclic adenosine monophosphate; GRK3, G protein-coupled receptor kinase.

activate different signaling cascades within a single brain region (Hjelmstad and Fields, 2003).

Dyn/KOR AND THE NEURAL CIRCUITS INVOLVED IN PAIN-INDUCED NEGATIVE AFFECT

Mesolimbic Circuitry

Recent studies on pain and depression show that decreased motivation in goal-directed behavior is a characteristic feature of pain-induced negative affect (Cahill et al., 2014; Ji et al., 2018; Liu et al., 2019). Thus, the mesolimbic circuitry, which is also known as the reward circuitry, may play a critical role in driving pain-induced negative affective states. This circuitry is composed of DA neurons in the ventral tegmental area (VTA) and their projections to forebrain limbic structures such as the nucleus accumbens (NAc) and prefrontal cortex (PFC).

VTA DA neurons receive strong dynorphin projections from the striatum, the lateral hypothalamus, the central nucleus of the amygdala (CeA), and the BNST (Fallon et al., 1985; Kaufling et al., 2017). Recently, Cahill's lab reported that chronic pain caused an increase in KOR mRNA (Oprk1) expression in DA neurons only in male mice and that pain-induced aversive states were increased by a KOR agonist in male but not female mice (Liu et al., 2019). The authors attributed the observed sex differences to complex hormonal and sex-chromosome factors, which are known to influence the depressive and anti-nociceptive effects of KOR in non-pain models (Russell et al., 2014; Abraham et al., 2018; Zhang et al., 2018). Liu et al. (2019) also reported that ablation of KORs from DA neurons using AAV-TH-cre virus in KOR loxP mice prevented pain-induced aversive states without affecting the sensory dimension of chronic pain (Liu et al., 2019). KORs are distributed in both the somatodendritic and terminal regions of VTA DA neurons (Tejeda et al., 2013). Activation of KORs located in the terminals of DA neurons inhibits DA release in efferents (Spanagel et al., 1992), but activation of KORs located in VTA cell bodies appears to have pathway-dependent effects, hyperpolarizing the dopaminergic projection to the mPFC and amygdala but not the projection to the NAc core (Margolis et al., 2006, 2008). Circuit DA dynamics are also shaped by Dyn/KOR-induced presynaptic inhibition of excitatory neurotransmission and GABA release onto VTA DA neurons (Graziane et al., 2013). Although many findings indicate that inhibition of the DA output is the mechanism underlying KOR agonist-induced

aversion, there is evidence to suggest that the recruitment of p38 MAPK signaling in DA neurons is critical. Conditional knockout of p38 signaling in DA neurons did not abolish the ability of a KOR agonist to inhibit DA release but did block KOR-mediated aversion (Ehrich et al., 2015). Indeed, DA release is not decreased in many pain paradigms (Navratilova et al., 2012; Xie et al., 2014), such as PGE2-induced hyperalgesia (Vergara et al., 2020), that produce aversive affect in behavioral tests. Therefore, the hypothesis that the recruitment of p38 MAPK signaling in DA neurons is more important than DA transmission alteration in KOR-mediated aversion seems to work in pain states, but further work is needed to identify the mechanism underlying the effects of the VTA Dyn/KOR system in pain-induced negative emotions.

Unlike in the VTA, which receives dynorphin projections from other nuclei, dynorphin is released locally in the NAc from medium spine neurons (MSNs). Massaly et al. (2019) reported an increase in local dynorphin tone in the NAc shell in inflammatory pain, resulting from pain-induced selective disinhibition of dynorphin-containing neurons. Similarly, higher levels of Oprk1 mRNA are observed in the NAc in animals with a spared nerve injury (Palmisano et al., 2017). It is noteworthy that the increase in KORs usually occurs during the early phase of neuropathy whereas downregulation of Oprk1 gene expression happens during the late phase, 2 weeks after surgery (Vergara et al., 2020), and may be attributed to internalization and densensitization of KORs. Furthermore, it has been demonstrated recently that KOR blockade in the NAc reverses preclinical measures of injury-induced aversion and anhedonia and that photogenetic activation of dynorphin-containing MSNs is sufficient to lead to negative affective states (Massaly et al., 2019). In paclitaxel-induced neuropathy, an injury-free model, a KOR antagonist injected into NAc reversed paclitaxel-induced anhedonia but not mechanical hypersensitivity (Meade et al., 2020). Together, these results suggest that pain-induced negative affect is mediated via recruitment of the NAc Dyn/KOR system.

Amygdala

The amygdala circuitry most relevant for pain-related functions includes the BLA, the central nucleus of the amygdala (CeA), and the intercalated cell clusters interposed between them (Thompson and Neugebauer, 2017, 2019). KORs are located on cells in both the BLA and the CeA, and dynorphin is released from distal projections as well as synthesized locally in the lateral subdivision of the CeA (CeL) (Kravets et al., 2015).

Previous studies have demonstrated that chronic pain promotes neuroplasticity mainly in the CeA (Carrasquillo and Gereau, 2008). Interestingly, modulation of the Dyn/KOR system in the CeA induces significant changes in pain-induced aversion (Nation et al., 2018; Navratilova et al., 2019) and KORs in the CeA have been linked to negative affective states associated with ongoing pain. Microinjection of a long-lasting KOR antagonist, nor-binaltorphimine (nor-BNI) into the right CeA before spinal nerve ligation in rats prevented neuropathic-induced conditioned place preference to intravenous gabapentin, suggesting that nor-BNI eliminated the aversiveness of ongoing pain (Nation et al., 2018). This effect was mediated by blocking the KOR-mediated disinhibition of CeA output neurons involved in neuropathic pain. Similarly, activation of the pathway from the lateral parabrachial nucleus (lPBN) to the CeA generates an aversive memory and dynorphin-expressing neurons are required in the process (Chiang et al., 2020). The lPBN is a major target of spinal projection neurons conveying nociceptive input to supraspinal structures. Most of the outputs from the CeA are GABAergic, coexpressing CRF and dynorphin, and project back to the lPBN. Optogenetic stimulation of the CeA–lPBN pathway suppresses acute pain and inhibiting it evokes pain behaviors in naïve animals, but the efficacy of this pathway is suppressed in chronic pain states (Raver et al., 2020). Hence, the CeA–lPBN circuit plays a negative feedback role in response to noxious stimuli under normal conditions but becomes inefficient in chronic pain states, and the Dyn/KOR system in the CeA is necessary for this role.

Dorsal Raphe Nucleus

The serotonergic system has been the focus of many studies on the relationship between pain and depression. The dorsal raphe nucleus (DRN) is one of the major sources of serotonin (5-HT) in the brain. Administration of complete Freund's adjuvant (CFA) results in sustained inflammatory pain and leads to depression-like behaviors. This model is widely used to induce comorbid pain and depression, which is characterized by depletion of 5-HT and its metabolism-related precursors in the brain (Zhang et al., 2016). Similarly, decreased levels of 5-HT are observed in the PFC in rats infused with a cocktail of inflammatory agents into the dura mater, which induces chronic headache and anxiodepressive-like behaviors (Zhang et al., 2017). Furthermore, recent human functional magnetic resonance imaging data show that functional connectivity between the DRN and the CeA is reduced in patients with comorbid depressive symptoms but not in patients with chronic pain only, compared with healthy controls (Zhou et al., 2019). Pharmacological and optogenetic results in animals further implicate a novel pathway involving 5-HT projections from the DRN to somatostatin-expressing neurons in the CeA in the comorbidity (Zhou et al., 2019).

Previous work indicates that administration of a KOR agonist into the DRN decreases extracellular 5-HT by approximately 30% (Fuentealba et al., 2010). It has been demonstrated that the Dyn/KOR system modulates serotonin transmission, especially in stress-related behaviors. First, KOR activation in the DRN inhibits the excitatory inputs onto serotoninergic neurons. Second, KORs increase postsynaptic G protein-gated inwardly rectifying potassium channel (GIRK) currents in the DRN. Lastly, KORs mediate the translocation of the serotonin transporter SERT via a p38 MAPK-dependent mechanism. Moreover, repeated stress exposure induces dynorphin release and KOR activation in 5-HT neurons in the DRN (Lemos et al., 2012). As a form of repeated stress, chronic pain may alter the DRN–CeA pathway by regulating the Dyn/KOR system in the DRN; however, more clinical and *in vivo* evidence is needed to fully elucidate the underlying mechanisms.

DRUG CANDIDATES AND FUTURE DIRECTIONS

There is no doubt that an analgesic with antidepressant and/or anxiolytic effects is optimal for patients with comorbid pain and mood disorders. However, this has proved to be an elusive goal for clinical and laboratory researchers for many decades. As one of the most commonly used categories of analgesic, opioids and opioid-based therapies may be the key to achieving this goal. Indeed, progress has already been made on several specific compounds targeting KORs for treatment of comorbid pain and psychiatric disorders (detailed in **Table 1**).

Biased G Protein KOR Agonists

Upon extracellular ligand binding, GPCRs usually undergo a conformational change that activates heterotrimeric G proteins, a process that is important for transmitting the required signals. The ability of agonists acting at the same GPCR to preferentially elicit different signaling pathways by stabilizing the receptor in a particular active conformational state is called "biased agonism" or "functional selectivity." The discovery of this phenomenon offers a therapeutic alternative to conventional full KOR agonism, which provides effective analgesia but at the cost of significant side effects including dysphoria, sedation, anxiety, and depression. There is consensus that KOR-coupled G protein signaling is the major pathway for the analgesic effects of KOR agonists whereas the arrestin-p38 MAPK cascade is required for aversion and other effects (Fields, 2011). Thus, biased G protein KOR agonists are promising compounds for the treatment of chronic pain.

Nalfurafine (TRK-820) was the first biased KOR agonist used in a clinical setting, for medication-resistant pruritus in hemodialysis patients (Kumagai et al., 2010). Recently, it has been reported that co-administration of nalfurafine with morphine beneficially modulates both the analgesic and rewarding properties of morphine in mice. Moreover, the dose of nalfurafine that produced a significant effect in the preclinical study (15 μg/kg) was similar to the antipruritic dose in mice, suggesting that the clinical dose may provide adequate analgesic synergy while avoiding significant antitherapeutic effects (Schattauer et al., 2017). Modification of the structural scaffold of salvinorin A (SalA), a potent KOR agonist, has produced several biased ligands, such as mesyl SalB, ethoxymethyl ether SalB (EOM SalB), and 22-thiocyanatosalvinorin A (RB-64). These are better biased G protein agonists for human KOR than SalA or U50,488H, another balanced KOR agonist (Simonson et al., 2015; Kivell et al., 2018). Mesyl SalB and EOM SalB produce longer-lasting analgesia in a warm-water tail-withdrawal rodent assay than SalA, without aversion, anxiety, or depressive-like effects (Ewald et al., 2017; Paton et al., 2017). Similarly, RB-64 induced analgesia in a hotplate assay in both wild-type and β-arrestin2 knock-out mice and, at a low dose, did not cause aversion-like responses (White et al., 2015). HS665 and HS666, which are diphenethylamine derivatives, exhibit great affinity for KORs and very weak partial agonism for β-arrestin-2 signaling. These compounds elicited a potent dose-dependent analgesic effect in the warm-water tail-withdrawal assay when administrated intracerebroventricularly (Spetea et al., 2012, 2017) and had no effect on locomotor behavior or aversion (Erli et al., 2017). A different study showed that both agents display analgesic action in an acetic acid writhing assay when injected subcutaneously, while demonstrating no motor impairments or sedation (Dunn et al., 2019). Collybolide (Colly) is a very potent biased agonist for treating comorbid pain and psychiatric disorders. Similar to SalA, it has an antinociceptive effect in the tail-flick assay and produces some aversion. However, unlike SalA, Colly exhibits slight antidepressant and anxiogenic effects in the forced swim and open-field tests (Gupta et al., 2016).

Peripherally Restricted KOR Agonists

Activation of peripheral KORs alone can produce a significant analgesic effect. Thus, the development of peripherally restricted KOR agonists that cannot cross the blood–brain barrier is a viable strategy for avoiding the side effects associated with activation of KORs in the CNS. However, many of the early compounds were ruled out because of insufficient antinociception; researchers were unable to identify effective compounds until recently.

Difelikefalin (CR845) and JNJ-38488502 (CR665) are peripherally restricted tetrapeptide KOR agonists that have shown promising results in preclinical studies, including a reduction in writhing behaviors and inflammatory pain in animal models (Hughes et al., 2013). CR845 is also effective in relieving abdominal pain and mechanical allodynia in a spinal nerve ligation model of neuropathic pain (Beck et al., 2019a). CR845 is currently in phase III clinical trials for the treatment of postoperative pain and uremic pruritus. One of the derivatives of CR665, termed JT09, is currently in development by JT Pharmaceuticals. 20 mg/kg JT09 administered via oral gavage in rats exhibited similar analgesic effects as 10 mg/kg morphine, but JT09 showed no sedative or pro-depressive effects in behavioral tests (Beck et al., 2019b). The pharmacodynamics of JT09 and its antinociceptive effects in chronic pain models are still being investigated. There is also a range of peripherally restricted derivatives of nalfurafine that have an increased number of hydrogen bond donors, and these have yielded promising results. These compounds produce dose-dependent anti-allodynic effects in the acetic acid writhing mouse model (Suzuki et al., 2017). Further work is planned to fully evaluate the antinociceptive potential of these compounds and assess the side effects. However, whether peripherally restricted KOR agonists can block pain-related depression is still unclear.

KOR Antagonists

Although it is well known that blockade of KOR activation prevents stress-induced aversive affects, the potential clinical use of KOR antagonists as antidepressants was not addressed until recently. Interestingly, KOR antagonists have been found to play an analgesic role in several injury-free pain studies. For example, stress produces allodynia in many injury-free models of cephalic pain. Microinjections of nor-BNI or CYM-51317 (a novel short-acting KOR antagonist) into the CeA in rats prevented this stress-induced allodynia (Xie et al., 2017).

TABLE 1 | Potential compounds for the treatment of comorbid pain and psychiatric disorders.

Compound	Function	Subjects and dose: pain treatment	Pain model/paradigm	Subjects and dose: aversion test	Aversive behavioral paradigm	References
Nalfurafine (TRK-820)	G-protein-biased KOR agonist	C57BL/6 mice 15 μg/kg	Warm-water tail-withdrawal			
Mesyl SalB	G-protein-biased KOR agonist	B6.SJL mice 1 mg/kg	Intraplantar formaldehyde (inflammatory pain model), warm-water tail-withdrawal	SD rat 0.3 mg/kg	Sucrose self-administration CPA CTA	Kivell et al., 2018
EOM SalB	G-protein-biased KOR agonist	C57BL/6J mice 0.1, 0.3 mg/kg	Warm-water tail-withdrawal	SD rat 0.1, 0.3 mg/kg	CPA EPM FST	Ewald et al., 2017; Paton et al., 2017
RB-64	G-protein-biased KOR agonist	C57BL/6 mice 3, 10 mg/kg	Hotplate analgesia	C57BL/6 mice 3 mg/kg	CPA	White et al., 2014; White et al., 2015
HS665	G-protein biased KOR agonist	C57BL/6J mice 10, 30 nmol, i. c.v.	Warm-water tail-withdrawal	C57BL/6J mice 10, 30 nmol, i.c.v.	CPA (have aversion)	Spetea et al., 2017
		C57BL/6J mice 30 nmol, i. c.v.	Acetic acid-induced writhing			Spetea et al., 2012
HS666	G-protein-biased KOR agonist	C57BL/6J mice 10, 30 nmol, i. c.v.	Acetic acid-induced writhing	C57BL/6J mice 30 nmol, i.c.v.	CPA	Spetea et al., 2012
Collybolide	G-protein-biased KOR agonist	C57BL/6J mice 2 mg/kg	Tail flick test	C57BL/6J mice 2 mg/kg	CPA FST EPM	Gupta et al., 2016
Difelikefalin (CR845)	Peripherally restricted KOR agonist	C57BL/6J mice 10 mg/kg	Spinal nerve ligation (neuropathic pain model)	C57BL/6J mice 10 mg/kg	OFT	Beck et al., 2019a
		Human clinical III phase	Postoperative pain			Gardell et al., 2008
JNJ-38488502 (CR665)	Peripherally restricted KOR agonist	SD rat 20 mg/kg	Acetic acid-induced writhing. hot plate analgesia	SD rat 20 mg/kg	OFT	Hughes et al., 2013
JT09	Peripherally restricted KOR agonist	SD rat 10 mg/kg	Acetic acid-induced writhing. hot plate analgesia	SD rat 20 mg/kg	CPA FST	Beck et al., 2019b
Nor-BNI	KOR antagonist	C57BL/6J mice 3 mg/kg, s.c 2.5 μg/lateral	Cephalic and extracephalic cutaneous allodynia, tail flick test	C57BL/6J mice SD rat	CPA FST EPM	Xie et al., 2017; Page et al., 2019
CYM-51317	KOR antagonist	C57BL/6J mice 20 mg/kg 1 μg/lateral	Cephalic and extracephalic cutaneous allodynia, tail flick test			Xie et al., 2017
CYM-53093	KOR antagonist	C57BL/6J mice 10 mg/kg	Tail flick-test migraine			Guerrero et al., 2019
Buprenorphine	Partial MOR agonist and KOR antagonist	Human (1–4 mg/d)	Postoperative moderate to severe pain	Older adults 0.4 mg/d	Montgomery–Asberg Depression Rating scale	Karp et al., 2014; Yovell et al., 2016

CPA, conditioned place aversion; CTA, conditioned taste aversion; EPM, elevated plus maze; FST, forced swim test.

Similarly, oral administration of CYM-53093 had a protective effect against migraine (Guerrero et al., 2019).

Using compounds which are both antagonistic to KORs and agonistic to other opioid receptors may be another good choice for treating comorbid pain and psychiatric disorders. Buprenorphine, a partial MOR agonist and KOR antagonist that provides long-lasting analgesia for chronic pain, is a promising candidate. It has been investigated for its anti-depression potential in preclinical studies and clinical trials and produced a rapid and sustained improvement in elders with resistant depression, even at a low dose. Furthermore, an early open-label study reported that buprenorphine alleviates negative symptoms in patients with treatment-refractory, unipolar, non-psychotic, major depression (Karp et al., 2014; Yovell et al., 2016).

CONCLUSION

Over the past decades, information has accumulated about the pathophysiological and pharmacological implications of the role of the Dyn/KOR system in the comorbidity of chronic pain and mood disorders. The Dyn/KOR system mediates pain-induced aversive states by regulating many aspects of emotion processing, including DA neurotransmission of the mesolimbic circuitry, the efficacy of the CeA–lPBN pathway, and the intrinsic excitability of 5-HT neurons. A new generation of biased KOR agonists together with new clinical medication strategies has led to a focus on KORs as a potential drug target for pain and psychiatric disorders with fewer side effects. These studies have increased our understanding of how the Dyn/KOR system

is involved in pain and mood disorders and have revealed promising therapeutic targets for the treatment of comorbid pain and psychiatric disorders.

AUTHOR CONTRIBUTIONS

M-JJ, JY, and Z-QG drafted the manuscript. CL critically edited the manuscript. LZ contributed substantially to the manuscript revision. All authors approved the manuscript in its final form.

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REVIEW
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Concepts of Neuroinflammation and Their Relationship With Impaired Mitochondrial Functions in Bipolar Disorder

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Bipolar disorder (BD) is a chronic psychiatric disease, characterized by frequent behavioral episodes of depression and mania, and neurologically by dysregulated neurotransmission, neuroplasticity, growth factor signaling, and metabolism, as well as oxidative stress, and neuronal apoptosis, contributing to chronic neuroinflammation. These abnormalities result from complex interactions between multiple susceptibility genes and environmental factors such as stress. The neurocellular abnormalities of BD can result in gross morphological changes, such as reduced prefrontal and hippocampal volume, and circuit reorganization resulting in cognitive and emotional deficits. The term "neuroprogression" is used to denote the progressive changes from early to late stages, as BD severity and loss of treatment response correlate with the number of past episodes. In addition to circuit and cellular abnormalities, BD is associated with dysfunctional mitochondria, leading to severe metabolic disruption in high energy-demanding neurons and glia. Indeed, mitochondrial dysfunction involving electron transport chain (ETC) disruption is considered the primary cause of chronic oxidative stress in BD. The ensuing damage to membrane lipids, proteins, and DNA further perpetuates oxidative stress and neuroinflammation, creating a perpetuating pathogenic cycle. A deeper understanding of BD pathophysiology and identification of associated biomarkers of neuroinflammation are needed to facilitate early diagnosis and treatment of this debilitating disorder.

Keywords: energy metabolism, mitochondria, bipolar disorder, oxidative stress, neuroinflammation, neuroprogression

INTRODUCTION

Bipolar disorder (BD) is a chronic and recurrent mood disorder characterized by cyclic episodes of depression and mania. Further, some patients may experience psychotic episodes during which there is a high risk of suicide. Depressive and manic episodes are often interspersed with periods of mood stability or euthymia (Goodwin and Jamison, 2007; Hoertel et al., 2013; Sigitova et al., 2017). Global prevalence has been estimated at approximately 1%–2%, but some estimates suggest that it may be as high as 4% (Kessler et al., 2005; Martinowich et al., 2009). BD can be divided into two subtypes, BD I characterized by severe manic and depressive episodes, and the less severe BD II characterized by hypomania and depression. A meta-analysis reported a centrally pooled lifetime prevalence of 1.1% for BD I and 1.2% for BD II (Clemente et al., 2015). Rates vary considerably across studies, however, possibly due to methodological differences. A recent epidemiological meta-analysis of 85 studies, including 67,373 adult patients from 44 countries, found a lifetime BD spectrum prevalence of 1.02%, relatively stable over three decades (Moreira et al., 2017).

The BD concordance rate is significantly higher between monozygotic twins than dizygotic twins, indicating a genetic influence (Barnett and Smoller, 2009). Studies have shown that BD shares pathogenic characteristics with a wide variety of other diseases, including metabolic, cardiovascular, and neurodegenerative diseases (Furman et al., 2019).

These and many other findings reviewed here, suggest that disease etiology is best explained by multiple interactions between environmental factors such as chronic stress and susceptibility genes (Goodwin and Jamison, 2007), altering the brain development, neuroplasticity, chronobiology, neurotransmission, and cell signaling pathways, ultimately leading to neuroinflammation, oxidative stress, and apoptotic cell death (Schloesser et al., 2008; Berk et al., 2011; Szepesi et al., 2018).

Progressive structural and biochemical changes in the prodromal and early stages of the disease, produce a slowly evolving clinical process called neuroprogression. The typical patient exhibits a slow decline in behavioral and cognitive functions associated with a weaker response to treatment (Berk et al., 2011; Borges et al., 2019). This slow progression prevents early diagnosis and rapid initiation of appropriate treatment. Finding an effective treatment regimen usually takes several years, resulting in substantial clinical impairment.

The main objective of this review article is to provide a better understanding of the pathophysiology of BD, especially the contributions of biomarkers such as neurotrophins, cytokines, oxidative stress, metabolic deficiencies, which are directly related to neuroinflammation (Fernandes et al., 2015).

We also seek to understand both the role of neuronal and glial cells, as well as the mitochondrial functions involved in neuroinflammation. These cells have high energy demands in relation to many other cell types, and the mitochondrial dysfunction produces the rupture of the electron transport chain (ETC), which leads to metabolic deficits, oxidative stress, cellular damage, and inflammation. Also, we summarized recent research conducted through biomarkers that were present in blood samples, which assisted in early diagnosis, and treatment response for better BD outcomes. Future research strategies based on these findings, processes, and their impacts on the evolution of the disorder, are discussed in detail below.

CHRONIC STRESS, NEUROINFLAMMATION, AND NEUROPROGRESSION, AS PATHOGENIC MECHANISMS UNDERLYING BIPOLAR DISORDER

A clear relationship has been established between chronic stress and neuropsychiatric pathology, including depression and BD, mediated primarily by dysregulation of the hormonal stress responses (Byrne et al., 2016; McEwen, 2017; Hei et al., 2019). Low levels of glucocorticoid released during acute mild stress can induce a compensatory increase in metabolism and enhance cognitive functions (Miller et al., 2009; Yaribeygi et al., 2017). For instance, glucocorticoid binding to high-affinity receptors can improve working memory and promote long-term memory consolidation by promoting dendritic growth and dendritic spine formation in the hippocampus, amygdala, and prefrontal cortex (Barsegyan et al., 2010; Liston et al., 2013). Under chronic stress, however, glucocorticoid levels decrease as a result of continuous or repetitive long-term stimulation (Hall et al., 2015). This is associated with reductions in glucocorticoid receptor expression and cortisol sensitivity, resulting in hypothalamic-pituitary-adrenal axis adaptation or habituation, particularly during emotional stress which has multiple long-term deleterious effects on neuronal functions, in different areas i.e., hippocampus, anterior cingulate cortex, prefrontal cortex, ventral striatum and insular cortex (Ulrich-Lai and Herman, 2009; Berk et al., 2013; Vieta et al., 2013; Jayasinghe et al., 2015; Rabasa et al., 2015).

Thus, when the challenges imposed by the social and physical environment appear unexpectedly and continuously exceed their limits of intensity and duration. Systems are activated which regulate homeostasis in higher levels of demands, which lead to the concept of allostasis. Thereby, allostasis is the ability to achieve stability by enacting compensatory responses to physiological and environmental stressors. The physiological repetition of allostatic cycles appears to accelerate the disease process (McEwen, 2000; Ganzel et al., 2010), including psychiatric disorders through a mechanism known as allostatic load (Rios, 2014). The allostatic load hypothesis was developed to explain the substantial clinical changes observed in the pathologies, and how these cumulative changes are reflected by the progression of the disease, leaving the cells and organs inefficient (i.e., pathogenic; McEwen, 2000; Grande et al., 2012). This concept has been transferred to brain diseases and has been referred to as neuroprogression (Berk et al., 2009, 2011; Salagre et al., 2018). Particularly in BD patients, the repeated episodes of depression and mania over decades, enhance the vulnerability to stress, further reducing the patients' recovery capacity and accelerating disease process, impairing several functions like the reduction of neural plasticity,

consequently reducing the memory capacity, irregular emotional responses, mood control, and decision making (Jansen et al., 2013; Vasconcelos-Moreno et al., 2017; Lacroix, 2019). This process can be better understood through the effects in the brain cells and their mitochondria, which have pro-inflammatory peripheral cytokine receptors, such as interleukins (IL)-6, IL-10, and tumor necrosis factor α (TNF-α), which respond by releasing second messengers, stimulating the production of more cytokines by the CNS. The initial stimulation may derive from damaged tissues releasing cytokines into the bloodstream, or by inflammatory stimulation of peripheral afferent neurons (Irwin and Cole, 2011; Fregnan et al., 2012; Zuccoli et al., 2017). Upon arrival in the brain, these proteins activate other cells and biochemical reactions, which enhance the allostatic load and can be modulated by multiple mechanisms like leukocytosis, a reduction in lymphocytes and natural killer cells, increased CD4+/CD8+ ratios, more proinflammatory cytokines (IL-1, IL-6, and TNF-α), cytokine receptor expression, and activation of the downstream nuclear factor kappa B (NF-κB) stress response pathway (Byrne et al., 2016; Gulati et al., 2016). At the cellular level, several of these effects are associated with altered calcium signaling. In chronic levels of cortisol, the expression of L-type calcium channels is upregulated by glucocorticoids promoting a greater Ca^{2+} influx into the cells promoting protease and phospholipase activation (Joëls et al., 2012, 2013; Merkulov et al., 2017). Also, when an overloaded calcium entry occurs, it leads to the opening of the mitochondrial permeability transition pore, and outer mitochondrial membrane permeabilization, respectively, facilitating the release of cytochrome c through the mitochondrial outer membrane, which triggers the caspase-3-dependent apoptosis cascade (Pereira et al., 2012; Perier et al., 2012; Di Meo et al., 2016). Furthermore, this increased calcium entry reduces ETC-coupled proton export, resulting in a reduced adenosine triphosphate (ATP) synthesis (Lin et al., 2012; Zhao et al., 2019). Furthermore, the extrinsic apoptosis pathway is triggered by the ligation of TNF-family death receptors at the cell surface. Receptor ligation can result in the recruitment of the Fas-associated death domain protein, which in turn binds procaspase-8 molecules, allowing autoproteolytic processing and activation of caspase-8, the principal effector of the extrinsic apoptosis pathway (Youle and Strasser, 2008; Machado-Vieira et al., 2009; Mitochondrial dysfunctions can also trigger excessive production of free radicals, leading to oxidative stress that eventually reduces metabolism and induces neuroplastic dysfunction, contributing to apoptosis by altering the structure of lipids, proteins, and DNA molecules (Machado-Vieira et al., 2007; Vakifahmetoglu-Norberg et al., 2017).

All of these elements are involved in the chronic stress response, which demonstrates their close relationship with the immune system, where all of them causes a decrease in the capacity for neuronal repair, and mitochondrial transport to synaptic regions *via* the cytoskeleton with further neuronal dysfunction and death (Mizisin and Weerasuriya, 2011; Lacroix, 2019). There is compelling evidence that BD arises through alterations in the synapses and critical circuit functions, rather than an imbalance of specific neurotransmitters mediating affective and cognitive functions (Martinowich et al., 2009; Scaini et al., 2020). Furthermore, prolonged metabolic dysregulation, deficient neurotrophin (NT) signaling, oxidative stress, and neuroinflammation, which may contribute to the increased frequency and severity of manic and depressive episodes, as well as other sequelae with age (Heneka et al., 2010; Kim et al., 2020). These neurological and behavioral abnormalities, in turn, will interfere with the patient's personal and professional life, leading to further stress-related pathogenesis. BD patients also exhibit an increase in psychiatric and medical comorbidities, which also may be associated with an imbalance of these mediators (Kapczinski and Streb, 2014; Rowland et al., 2018).

Increased levels of proinflammatory cytokines in the CNS stimulate the activation of immune cells, including macrophages, monocytes, and microglia. Rising inflammatory cytokines in the CNS appear to depend on the activation of microglia (McEwen, 2017).

The role of microglia and the participation of proinflammatory mediators in neuroinflammation will be discussed from this point forward. The CNS hosts a heterogeneous population of resident myeloid-derived immune cells that regulate communication between the nervous, vascular, and immune systems. Most prominent among these are the parenchymal microglia, which account for up to 16% of the total cell number in some areas of the human brain (Norden and Godbout, 2013). Microglia perform essential homeostatic functions under non-pathological conditions, including regulation of neural circuit development (Squarzoni et al., 2014) through the release of neurotrophins such as brain-derived neurotrophic factor (BDNF; Parkhurst et al., 2013), clearance of apoptotic cells and cellular debris, and synaptic pruning (Paolicelli et al., 2011). Microglia are also critical regulators of neuroinflammation in response to brain trauma and various pathogenic insults (Gomez-Nicola et al., 2014). Until recently, only circulating monocytes were thought to replenish tissue macrophage populations, including CNS microglia. However, new research suggests the presence of two ontogenetically and genetically distinct myeloid populations of microglia and nonparenchymal macrophages in the meninges, perivascular spaces, and choroid plexus (Jakubzick et al., 2013; Davies and Taylor, 2015; Herz et al., 2017). In rodents, microglial progenitors derived from the yolk sack appear on an embryonic day (E) 8.5 (Ginhoux et al., 2010; Gomez Perdiguero et al., 2015) distributed in the brain before birth, and remain a stable population throughout life. In contrast, other CNS macrophages likely originate from monocytes derived from the bone marrow (Ajami et al., 2011; Kierdorf et al., 2013), are short-lived after birth and show rapid turnover through proliferation and apoptosis (Aguzzi et al., 2013; Prinz and Priller, 2014), which renews the entire population several times over a lifetime (Askew et al., 2017). Rodent cell transplantation experiments (Hickey et al., 1992) and observations following bone marrow transplantation (Yang et al., 2013; Barr et al., 2015) also indicate that some monocytes in the blood and perivascular macrophages can infiltrate into the CNS parenchyma (Mildner et al., 2007; Kierdorf et al., 2015). Despite their distinct origins, CNS microglia and macrophages are morphologically similar

and share certain functions. However, microglia cannot be replaced by monocyte-derived macrophages due to their specific gene expression patterns and unique functions (Goldmann et al., 2013; Ginhoux et al., 2016; Prinz and Priller, 2017). In addition to microglial cells, nonparenchymal and endothelial cells regulate neural-immune function by maintaining the BBB, promoting angiogenesis, regulating the composition of the cerebrospinal fluid, and controlling vascular tone (He et al., 2016). Various dendritic cells, mast cells, monocytes, and granulocytes complete the CNS immune system (Kierdorf et al., 2015; Goldmann et al., 2016). Mast cells are one of the few cells that migrate to the CNS under both physiological and pathological conditions, where they reside in the neuronal parenchyma (Sayed et al., 2010) and function as pathogen sensors and modulate inflammation by recruiting other immune cells to specific target regions (Skaper et al., 2013). Many of these cells acquire anti-inflammatory or pro-inflammatory phenotypes (Brendecke and Prinz, 2015), and it is the balance between these phenotypes that determines overall neuroinflammatory status and the progression of neuroinflammatory diseases (Goldmann et al., 2016). Even in their inactivated resting state, microglia continually search for signs of potential threats to the CNS (Hellwig et al., 2013). Multiple pathways are activated by chemical signals from infection, trauma, endogenous and exogenous toxins, and the loss of constitutive anti-inflammatory signals. Studies have shown that microglia and brain macrophages can differentiate into two distinct phenotypic groups, the classically activated (M1) and alternatively activated (M2) populations (Chawla, 2010; Geissmann et al., 2010). These reactive phenotypes have distinct protein and non-coding mRNA expression profiles, release unique cytokines and chemokines, and have different phagocytic activities. The reactive behavior of M1 microglia can eliminate the initial activation trigger (such as a pathogen) with or without the support of other resident or invasive immune cells. This loss of the pathogenic stimulus leads to a more repair-oriented microglial profile and eventual reversion to the initial resting state (Arcuri et al., 2017; Tohidpour et al., 2017). Thus, the microglia produce an immune response during inflammatory conditions, moderating potential damage to the CNS and aiding in tissue repair and remodeling (Kingwell, 2012; Hellwig et al., 2013). Furthermore, in the early stages of diseases, symptoms may be followed by microglial polarization to M1 (Duffy et al., 2010; Yutaka and Kenji, 2014; Ginhoux and Guilliams, 2016). This M1 phenotype can produce proinflammatory cytokines and oxidative metabolites that cause additional damage, such as TNF-α and IL-6 and IL-1 (Colton, 2009; Miller and Raison, 2016). Activation of the M2 phenotype by IL-4, IL-13, or IL-10 (Nguyen et al., 2011; Nakagawa and Chiba, 2014) negatively regulates M1 function, thereby suppressing inflammation and promoting tissue repair and wound healing, consequently attenuating symptoms and restoring tissue homeostasis (Kawabori and Yenari, 2014). In certain chronic conditions, however, some cells may not return to a complete resting state, whereas others remain post-activated microglia. If M2 microglia polarization is insufficient, M1 microglial functions are maintained and induce sustained inflammation and progressive neural network dysfunction. In turn, symptom severity may gradually increase according to the frequency of M1 polarization (Yutaka and Kenji, 2014; Bachiller et al., 2018). These cells may maintain subtle changes, such as transcriptional activity, that modulate their sensitivity to anti-inflammatory signals or alter responses to subsequent stimulation. Sustained M1 activity may even lead to neuronal degeneration (Arcuri et al., 2017). A recent study reported that there was an M1 dominance in one of three BD patients during the manic state, and a downregulation of M2 markers during the manic state in all three patients, suggesting that the M1/M2 balance may indeed contribute to BD symptoms. The researchers showed that the gene profiling patterns are different between manic and depressive states (Ohgidani et al., 2017).

However, while activated microglia have demonstrated neurotoxic effects, responses may be very different *in vivo* compared to the commonly used *in vitro* models (Hellwig et al., 2013) due to the absence of inhibitory factors such as CD200, CX3CL1, CD22, and CD172, which maintain microglia attenuation *in vivo* (Ransohoff and Cardona, 2010; Prinz et al., 2011). Blocking even one of these inhibitory factors results in profound changes in microglial reactions, often causing a disproportionate immune response and occasionally cytotoxic responses (Hoek et al., 2000; Cardona et al., 2006). With all that, neurons may be damaged or functionally impaired when microglial activation is dysregulated, and microglia-mediated inflammation is intense, as observed in chronic brain pathologies. This inflammatory neuronal damage can contribute to the progression of neurological disease, and possibly psychiatric diseases such as BD (Perry et al., 2010; Kettenmann et al., 2011; von Bernhardi et al., 2015).

ENERGY DEFICITS IN NEURONAL MITOCHONDRIA AND ITS POSSIBLE RELATIONSHIP TO PSYCHIATRIC DISORDERS

Neurons contain large numbers of mitochondria to supply the energy required for the maintenance of ion gradients and electrical signaling, neurite growth, long-distance axonal transport (mitochondria to distal synapses), calcium homeostasis, and calcium signaling. Neurons are highly energy-demanding cells. A single cortical neuron at rest consumes approximately 4.7 million ATP molecules per second to execute various biological functions, including the maintenance of ionic gradients critical for electrophysiological signaling. In the human brain, the ATP utilization rate is three times higher in gray matter than white matter (Zhu et al., 2012, 2018), and gray matter neurons are responsible for approximately 20%–25% of all systemic oxygen and glucose consumption (Attwell and Laughlin, 2001). Proper mitochondrial functions are, therefore, critical for neural purposes.

Although the entire neuron requires energy, some sites display higher energy demand, including presynaptic and postsynaptic terminals that mediate neurotransmission, active growth cones or axonal branches, which regulate short- and long-term plasticity, and also Ranvier's nodes, where the

transmembrane ion flux is the highest (Zhang et al., 2010; Sheng and Cai, 2012). This is only possible because mitochondria are highly dynamic, and the relevance of these dynamic processes to BD and other psychiatric disorders is related to the constant changes in the mitochondrial number. It produces an altered mitochondrial distribution and a defective transport. The rapid movement of axonal mitochondria is a primary mechanism, underlying spontaneous and neural activity-dependent synaptic remodeling, being altered under certain conditions, such as stress and axonal trauma (Cataldo et al., 2010; Sheng and Cai, 2012; Sun et al., 2013; Chu, 2019). Sustained local mitochondrial energy production in the presynaptic region is critical for synaptic vesicle release. For instance, a drop in ATP levels in hippocampal synaptosomes reduces synaptic vesicle release and alters the cytosolic calcium concentration (Ivannikov et al., 2013). The postsynaptic site also has extensive energy requirements. A combined proteomics and mass spectroscopy study by Föcking et al. (2016) found high cytoskeletal and signaling protein densities in the postsynaptic region, facilitating the movement of receptors and activating complexes critical for standard synaptic transmission and plasticity. Aberrant synaptic plasticity is implicated in neuropsychiatric disorders such as schizophrenia and BD and may stem from mitochondrial dysfunction and reduced metabolism (Akula et al., 2015; Forero et al., 2016). As shown in **Table 1**, the mitochondrial protein synthesis combined involves a total of 37 nuclear DNA (nDNA), and mitochondrial DNA (mtDNA) genes. Many of these proteins are involved in both oxidative phosphorylation (OXPHOS) and ETC, and any change in genes can drastically interfere with metabolism (Björkholm et al., 2015; Garcia et al., 2017; Kang et al., 2018). Thus, multiple lines of evidence implicate mitochondrial dysfunctions in BD, including the ∼20-fold higher incidences of BD symptoms in patients with mitochondrial diseases. The research reported the identification of mitochondrial DNA deletions, polymorphisms in some BD cases, aberrant up- or downregulation of various mitochondrial genes, BD-like behavioral phenotypes in mouse models with mitochondrial gene mutations. Furthermore, there were differences in mitochondrial morphology, distribution, and metabolite levels between BD patients and controls (Kato, 2017).

Mitochondria produce ATP from metabolites through two continuous biochemical processes, the tricarboxylic acid cycle (TCA) and OXPHOS (Vidyasagar, 2015). ATP generation within the mitochondrial matrix requires interconnected processes (Cooper and Hausman, 2006; Kühlbrandt, 2015). First, fatty acids and the glycolytic degradation product pyruvate are converted into acetyl-CoA *via* matrix enzymes (Cronan and Laporte, 2005) and enter the TCA cycle (**Figure 1**), which generates electron-rich NADH and $FADH_2$ as sources for ETC complexes I and II, respectively (Enríquez, 2016). The electrons are transferred to Coenzyme Q10, which is essential due to its antioxidative properties (Bentinger et al., 2010). Coenzyme Q10 transfers the electrons to Complex III, where they are transported to Complex IV by cytochrome c (Alvarez-Paggi et al., 2017). In Complex IV, electrons are transferred to molecular oxygen to form water (Enríquez, 2016; Milenkovic et al., 2017). Finally, hydrogen is pumped through Complex V to store energy for ATP formation from ADP and inorganic phosphate, which is coupled to controlled re-entry of protons in the mitochondrial matrix (**Figure 2**; Walker, 2013; Angrimani et al., 2015).

TABLE 1 | Structural codifications of Oxidative Phosphorylation System (OXPHOS) complexes from nDNA and mtDNA.

Structural codification of OXPHOS complexes					
Complex	**I**	**II**	**III**	**IV**	**V**
nDNA encoded polypeptides	≅ 38	4	10	10	16
mtDNA encoded polypeptides	7	0	1	3	2

The interest of neuropsychiatry in the TCA cycle and oxidative stress focused on the studies related to the production of brain energy. The TCA cycle plays an important role since it is responsible for the reactions that generate the substrates for OXPHOS that occur in ETC. The expression of levels or activities of several TCA enzymes are altered in the brains of BD patients, which may contribute to both neuronal energy deficits and oxidative stress (Blass and Brown, 2000; Zuccoli et al., 2017). Research with bipolar patients and animal models observed a reduction in TCA cycle enzymes (Lee et al., 2007; Valvassori et al., 2013). In oxidative stress, it is essential to note that mitochondria are the primary source of free radicals, and are generated mainly through the ETC, during the energy production from glucose and oxygen, it generates oxidative stress (Barbosa et al., 2010; Mandavilli et al., 2018). Initially, we will describe the main processes, functions, and losses in oxidative stress, and later, the changes that occur in BD, which are related to the section on biomarkers.

Free radicals are molecules that have at least one unpaired valence electron, resulting in chemical instability and high reactivity with other molecules being continuously produced under physiological conditions (**Figure 3**). However, the free radicals, both the reactive oxygen species (ROS) and reactive nitrogen species (RNS), are derived from both endogenous sources (mitochondria, peroxisomes, et cetera), and exogenous sources (alcohol, heavy metals, et cetera; Phaniendra et al., 2015).

The physiologically and pathologically relevant free radicals include; superoxide ($O_2 \bullet -$), hydroxyl ($OH\bullet$), nitric oxide ($NO\bullet$), peroxyl-lipids ($LOO-$), and hydrogen peroxide (H_2O_2), and they are formed *via* enzymatic and non-enzymatic reactions (Reynolds et al., 2007; Collin, 2019). Superoxide is the leading free radical species formed by the ETC, and thus, production is enhanced in metabolically active neurons. The $OH\bullet$ is the most reactive biological species and can damage for instance ETC complexes I, II, and III. It can also lead to Fe-S electron center complex malfunction in the TCA cycle and affect mtDNA, leading to further oxidative stress resulting in a reduction in mitochondrial energy production (Federico et al., 2012; Ghezzi and Zeviani, 2012; Voets et al., 2012; Kausar et al., 2018). In contrast, hydrogen peroxide is not strongly reactive but can be toxic due to its long half-life, damaging the membrane permeability (Barreiros et al., 2006).

In healthy cells and tissues, multiple enzymatic and non-enzymatic antioxidant defense systems can reduce the damage caused by free radical production. However, most ROS are neutralized by endogenous enzymatic antioxidants which consist of diverse proteins that metabolize free radicals,

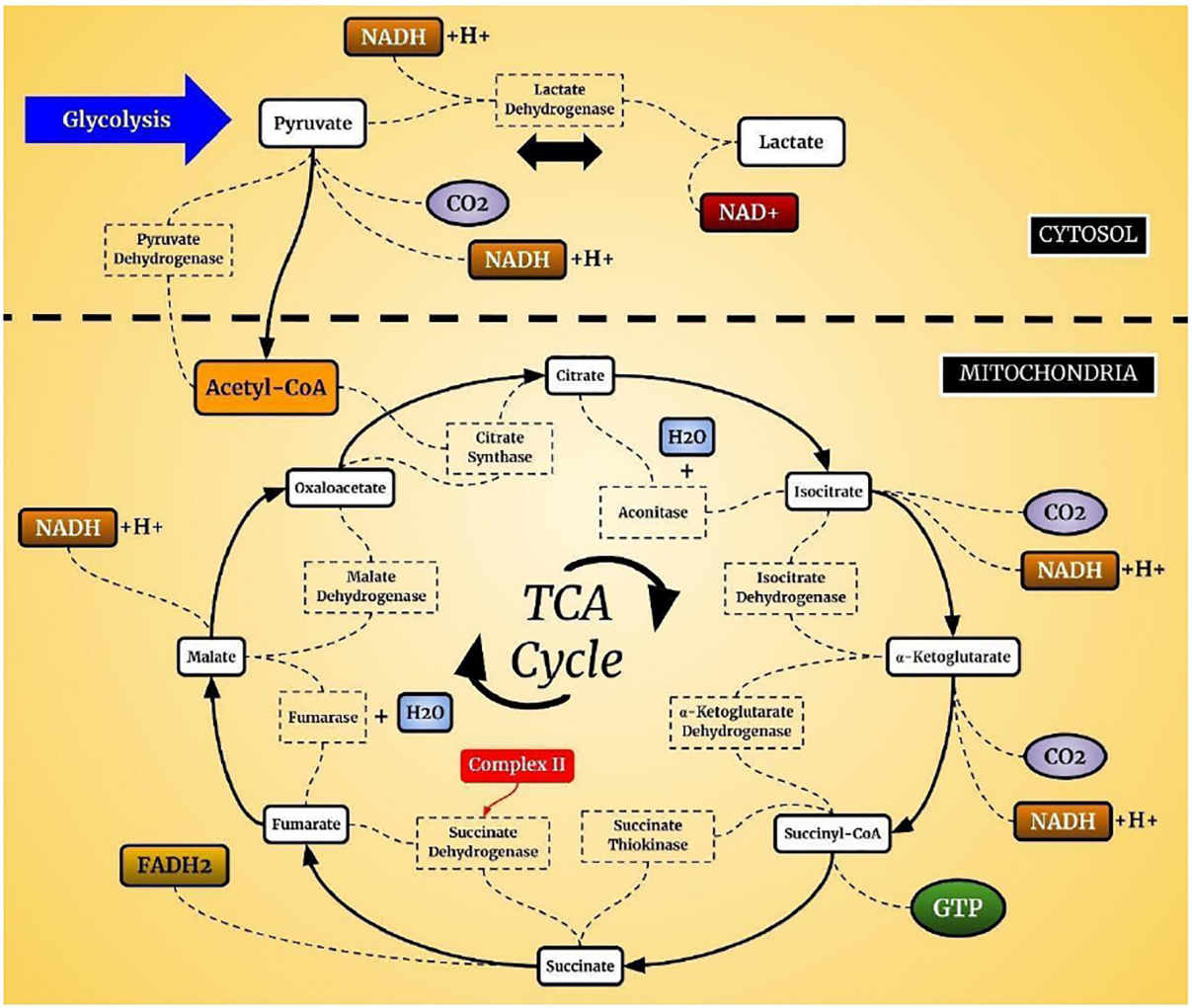

FIGURE 1 | Tricarboxylic acid cycle (TCA) cycle. In aerobic organisms, glucose is oxidized to CO_2 and H_2O. The pyruvate present in the cell cytosol is oxidized to acetyl-CoA which can enter the TCA cycle. This cycle is composed of a complex of enzymes located in the mitochondrial cytosol of eukaryotic cells.

reduce oxidized molecules, and peroxidized lipids, being the best known, the superoxide dismutase (SOD), catalase (CAT), GSH S-transferase, y-glutamylcysteine synthetase, GSH peroxidase (GSH-Px), and GSH reductase (Jeeva et al., 2015; Kurutas, 2016). When free radical production is greater than the endogenous antioxidant capacity, it leads to oxidative stress (Barreiros et al., 2006; Barbosa et al., 2010). Among the most pathogenic results of oxidative stress in neurons, is self-perpetuating membrane lipid peroxidation, which results in reduced membrane fluidity and barrier function (Ademowo et al., 2017). SOD acts as the primary protective enzyme against oxidative stress and DNA damage in mitochondria by catalyzing the dismutation of $O_2 \bullet -$ into H_2O_2 and O_2 (Gill and Tuteja, 2010; Krishnamurthy and Wadhwani, 2012). In turn, H_2O_2 is converted to H_2O and O_2 in most tissues by CAT (Barbosa et al., 2010). At low concentrations, H_2O_2 regulates several physiological processes, however, at higher concentrations, it damages cells by reacting with cellular iron to form hydroxyl radicals. Therefore, CAT is critical for limiting H_2O_2-induced damage (Ighodaro and Akinloye, 2018). GSH-Px inhibits lipid peroxidation, thereby preventing loss of membrane function. Like CAT, it acts by catalyzing the reduction of H_2O_2 or RHO_2 to H_2O by GSH, which is concomitantly oxidized to form the disulfide-bonded dimer GSSG (Espinosa-Diez et al., 2015).

It is important to highlight that the brain is particularly vulnerable to oxidative damage because it uses a high oxygen utilization rate, associated with weak defense of antioxidants, and a constitution rich in lipids, favoring the oxidative damage in neuronal cells (Salim, 2017). Thus, alterations in antioxidants and various oxidation products suggest a possible link between oxidative stress and BD. Evidence demonstrates that ROS act as essential second messengers in innate and adaptive immunity (West et al., 2011; Kamiński et al., 2013), stimulating proinflammatory cytokine generation (including IL-1B, IL-8,

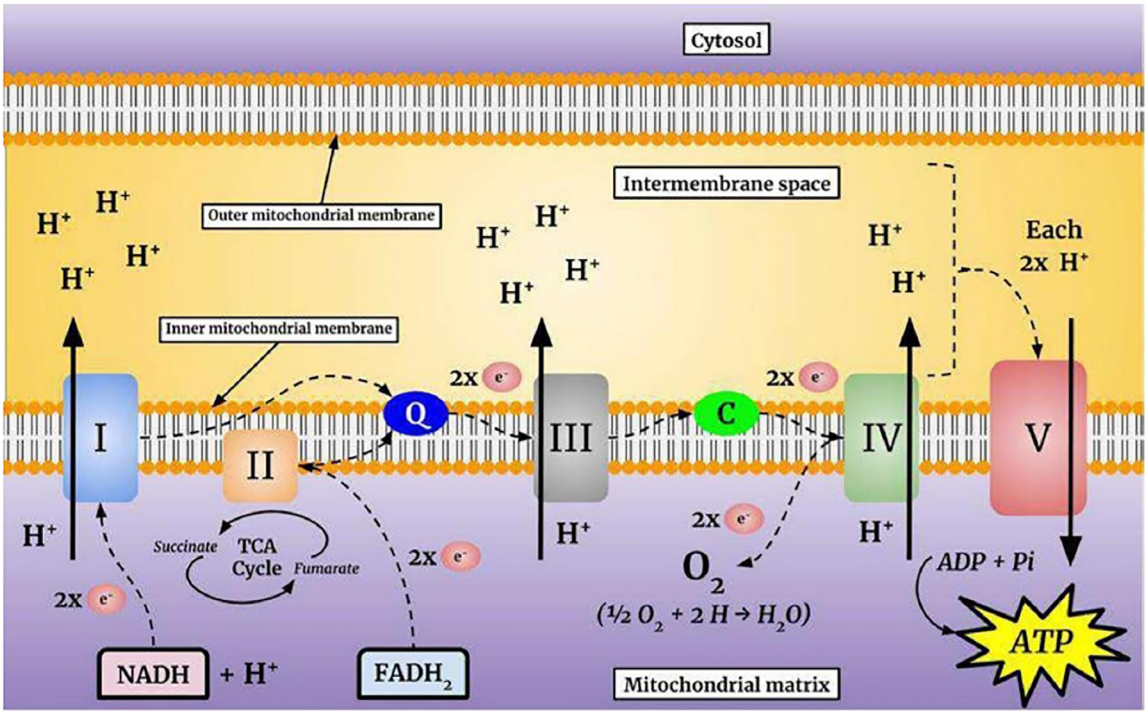

FIGURE 2 | The mitochondrial electron transport chain (ETC). The flow of electrons through the complexes is energetically coupled to the pumping of protons into the intermembrane space. This process produces an electrochemical gradient that stores the energy necessary for adenosine triphosphate (ATP) synthesis.

TNF-α, and interferons) during the immune response to control pathogens and repair tissue damage (Chen and Nuñez, 2010; Mittal et al., 2013). Therefore, in BD, a probable hypothesis is that a higher oxidative stress load is generated by a fundamental disturbance in mitochondrial functions, aggravating the disease. Based on these findings, improved mitochondrial functions is a potentially promising strategy for BD treatment.

BIOMARKERS RELATED TO NEUROINFLAMMATION IN BIPOLAR DISORDER

Collectively, the findings mentioned above indicate that BD should be treated as a multisystem inflammatory disease. An analysis of biomarkers for inflammation and oxidative stress showed that patients with acute BD onset have a significantly higher systemic toxicity than healthy controls, although not as severe as sepsis (Pfaffenseller et al., 2013). Although consequent allostatic overload associated with neuroinflammation in BD patients has been present, a causal link between systemic toxicity and biomarkers has not yet been established. As such, there is an increasing interest in identifying peripheral biomarkers that could function as indicators for systemic and cellular toxicity in BD (Kapczinski et al., 2011; Frank et al., 2014). Specific biomarkers or combinations may be associated with the degree of disease activity during active periods or remission. Notably, some systemic markers have already been implicated as mediators of BD allostasis, and in neuroinflammation (Juster et al., 2013). Thus, such studies have improved our understanding of the disease's activity and progression, providing clues to new novel therapeutic targets.

While there is still no reliable set of biomarkers for early diagnosis, many show promise for disease detection and treatment evaluation. These biomarkers fall into three categories: (1) imaging signs; (2) genetic loci; and (3) metabolic molecules. Category 3, includes various substances that are derived from neuronal and glial cells, such as the neurotrophins BDNF, glia-derived neurotrophic factor (GDNF), and neuronal growth factor. The pro-inflammatory cytokines IL-6, IL-1, and TNF-α, TCA markers such as citrate synthase, succinate dehydrogenase, and malate dehydrogenase, and oxidative stress-related markers including SOD, CAT, GLUT-Px, 3-nitrotyrosine, and products of lipid peroxidation (Thiobarbituric Acid-Reactive Substance; de Sousa et al., 2015; Scaini et al., 2016). In this review article, we will focus mainly on the last group (metabolic molecules), associating the alterations found in BD and their relationship with neuroinflammation.

Neurotrophins

Neurotrophins are small secreted proteins that promote multiple neuronal responses through surface receptor binding and activation of several downstream kinase signaling pathways. More than 50 neuronal growth factors are expressed in the mammalian brain. The NT family members BDNF,

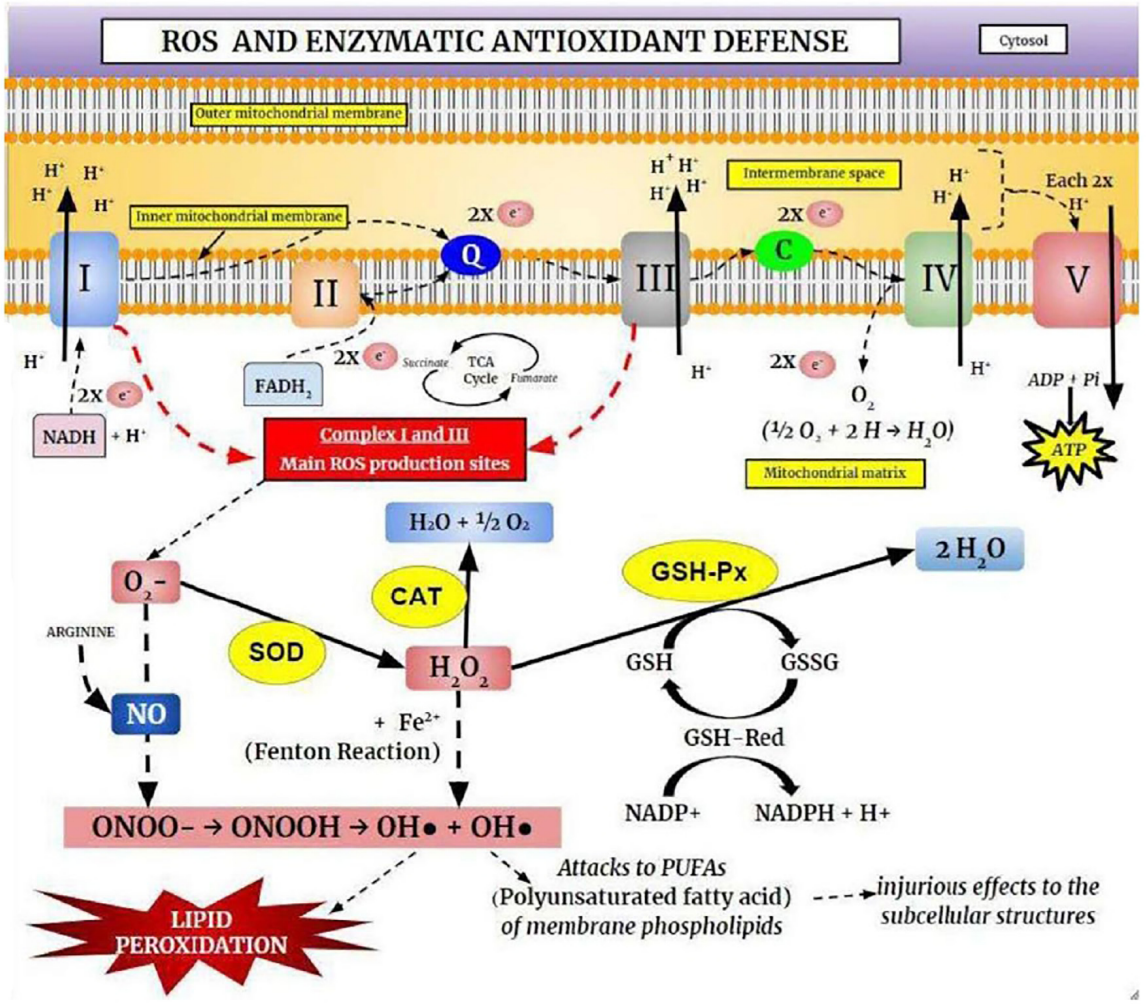

FIGURE 3 | The Oxidative Phosphorylation System (OXPHOS) and the enzymatic antioxidant defense. Electrons derived from cellular metabolism reach Complexes I or II through NADH or $FADH_2$, respectively. These electrons are then transferred to Coenzyme Q10 (ubiquinone), which carries electrons from Complexes I or II to Complex III. In Complex III, electrons are displaced from cytochrome b to cytochrome c and consequently transferred to Complex IV (cytochrome c oxidase), where they reduce O_2. These electrons are transported through mitochondrial protein complexes and are coupled to proton pumping into the intermembrane space. Complex V uses the generated electrochemical gradient for ATP synthesis. Complexes I and II are responsible for the production of superoxide anion (O_2-), which is removed by the antioxidant enzyme superoxide dismutase (SOD). This process produces hydrogen peroxide (H_2O_2), which is removed by catalase (CAT) and glutathione peroxidase (GSH-Px) enzymes. Superoxide and H_2O_2 can be converted into highly reactive hydroxyl radicals (OH·), causing lipoperoxidation and cellular injury.

GDNF, NGF, NT-3, and NT-4/5 increase cell survival by stimulating axonal regeneration following injury and inhibiting apoptotic protein cascades, and promote multiple forms of neurite and synaptic plasticity (Machado-Vieira et al., 2009; Pereira et al., 2012). BDNF activates two distinct receptors, the NT p75 receptor, and the Trk tyrosine kinase receptor. These two receptors can have opposing actions depending on ligand availability and cellular context (Mocchetti and Brown, 2008; Sasi et al., 2017). Both receptors regulate development, survival, repair, cortical dendritic growth, and plasticity as observed, for example, in the visual cortex and its connections (Huberman and McAllister, 2002; Sutton and Schuman, 2006). The p75 receptor signals mainly through stress-associated pathways such as JNK, p53, and NF-κB, while Trk receptors activate the Akt and mitogen-activated protein kinase/extracellular regulated kinase (MAPK/ERK) pathways. Activation of the Trk receptor by BDNF phosphorylates target proteins such as phospholipase C, phosphatidylinositol-3 kinase (PI3K), and ERK1/2 (Kaplan and Miller, 2000; Park and Poo, 2013). The MAPK/ERK pathway initiates a cascade that inhibits pro-apoptotic proteins and increases the expression and phosphorylation (activation) of the transcription factor nuclear cAMP response element (CREB), which upregulates the expression of neurotrophic/neuroprotective proteins such as Bcl-2 and BDNF (Machado-Vieira et al., 2009; Benito and Barco, 2010). Chronic cell stress can result in the dysregulation

of any component of the BDNF–MAPK/ERK–CREB pathway. For example, overstimulation by cortisol can negatively regulate CREB phosphorylation and subsequently decrease the transcription of NT genes such as BDNF (Kandel, 2001; Carlezon et al., 2005). This fact is especially vital in psychiatric illnesses and may contribute to the pathophysiology of BD (Berk et al., 2009; Tramontina et al., 2009).

Out of these, BDNF is the most widely distributed and is also the most studied in BD. The initial meta-analyses have reported reduced serum BDNF in BD patients during manic or depressive states, compared to euthymia. These discoveries have been found both in the serum of living BD patients, as well as in postmortem neurons (Knable et al., 2004; Sen et al., 2008; Fernandes et al., 2009; Lin, 2009). However, other meta-analyses and longitudinal studies have demonstrated that the reduced BDNF levels associated during BD manic and depressive phases were responsive at clinically useful drugs like lithium can elevate BDNF expression in the brain (Lang et al., 2007; Yang et al., 2009; Schmidt et al., 2011). More recently, Fernandes et al. (2015) performed a systematic review and meta-analysis evaluated serum and plasma BDNF levels in BD including a total of 52 studies with 6,481 participants, showing that, compared to healthy controls, peripheral BDNF levels are reduced to the same extent in manic and depressive episodes, while BDNF levels are not significantly altered in euthymia. The researchers showed the BDNF levels were negatively correlated with the severity of both manic and depressive symptoms. However, they found no evidence for a significant impact of illness duration on BDNF levels. Also, in plasma peripheral, BDNF levels increase after the successful treatment of an acute manic episode, but not of a depressive one demonstrating that BDNF is a potential biomarker of disease activity in BD, but not a biomarker of the stage (Panaccione et al., 2015; Roda et al., 2015). While it is not always clear whether reduced BDNF is a cause or consequence of BD-related pathology, there is a suggestive association between changes in brain BDNF levels and BD. The reduced serum BDNF in the brains of BD patients exhibits a variety of gross and fine morphological changes that become more pronounced with repeated episodes and disease duration (Tramontina et al., 2009; Olsen et al., 2013). For instance, reductions in the density of oligodendrocytes and myelination in BD patients showed brain white matter abnormalities have been observed in subgenual prefrontal cortex layer VI, caudate nucleus, and the hippocampus along with signs of necrosis and apoptosis (Mechawar and Savitz, 2016; Ganzola and Duchesne, 2017). Also, reduced neuronal somal size, increased somal density, and reduced dendritic spine density have been observed in the anterior cingulate cortex of BD patients. These changes may explain the associated impairments in cognition and judgment (Vostrikov et al., 2007; Konopaske et al., 2014).

As reported above, both cross-sectional and longitudinal studies have indicated that administration of antidepressants and mood stabilizers such as lithium and valproate has normalized BDNF, promoting so, a neuroprotection stress-induced, protecting the cells through anti-apoptotic pathways activation (Colucci-D'Amato et al., 2020). It can also increase the gray matter promoting neurogenesis in the subventricular zone of the lateral ventricle and subgranular zone of the hippocampal dentate gyrus, and improve cognitive functions such as learning and memory, both in animal and human studies (Sassi et al., 2002; Gould, 2007; Kempton et al., 2008; Machado-Vieira et al., 2009; Hashimoto, 2010; Corena-McLeod et al., 2013; Yu and Greenberg, 2016). However, the utility of BDNF as a biomarker during the early or late phases of the disease remains to be determined in longitudinal studies. Further studies are also needed to identify whether BDNF modulation can reduce acute episodes, and promote the possibility for patients to return to euthymia.

Other neurotrophins and neurotrophic factors are also altered in BD patients, reinforcing the hypothesis that impairments in neuroplasticity are involved in pathophysiology (Scola and Andreazza, 2015). Both NT-3 (Fernandes et al., 2010) and NT-4/5 (Walz et al., 2009) were increased during manic and depressive episodes compared to euthymic patients or healthy controls. However, studies on GDNF changes are conflicting. Barbosa et al. (2011b) found increased plasma GDNF levels in euthymic patients compared to manic patients and controls, while Rosa et al. (2006) observed increased GDNF levels in manic and depressive patients, but not in euthymic patients, compared to controls. In another study, GDNF serum levels were reduced in patients during manic and depressive episodes but increased after mood stabilizer treatment (Zhang et al., 2010). Additional studies are needed to assess whether peripheral GDNF levels correlate with CNS levels.

Inflammatory Cytokines

Both peripheral immune cells and resident brain cells, such as astrocytes, oligodendrocytes, and microglial cells, are associated with elevated pro-and anti-inflammatory cytokines (Barbosa et al., 2011a). The imbalance between them, have been implicated in neuroinflammation, causing toxicity and apoptosis of neurons and glial cells (Dong and Zhen, 2015; Réus et al., 2015; Muneer, 2016), which is associated with neuroprogression in BD, as well as other psychiatric diseases (Kato et al., 2013).

The prevailing hypothesis is that the immune system is chronically activated in BD mainly through microglial activation, which leads to an imbalance of pro-and anti-inflammatory cytokines and chemokines, which in turn can deregulate moods. This hypothesis arose after a significant number of patients with hepatitis C (treated using IFN-α), experienced depressive or manic symptoms (Hoyo-Becerra et al., 2014). It is not clear how peripheral cytokines affect inflammatory processes in the CNS since they do not readily cross the BBB under physiological conditions. Furthermore, postmortem studies have shown an increase or decrease in various pro- and anti-inflammatory factors in the prefrontal cortex, hippocampus, and cingulate gyrus of BD patients (Rao et al., 2010; Sneeboer et al., 2019). Also, many of the changes in cytokine levels found among bipolar patients, are similar to those observed in schizophrenia and major depression during acute and chronic disease phases (Hope et al., 2009; Momtazmanesh et al., 2019). Numerous studies have evaluated serum concentrations of cytokines (IL, IFN, TNF), growth transforming factors, and chemokines in BD patients. Research has pointed to an increase

in mainly proinflammatory factors i.e., IL-1β, IL-6, and TNF-α (Forrest et al., 2018; Formanova et al., 2019; Kany et al., 2019; Pawluk et al., 2020). On the other hand, several studies have shown that anti-inflammatory cytokines (IL-4, IL-10, IL-13, IGF-1, TGF-β) increase BDNF release and inhibit microglial proinflammatory activity, resulting in increased synaptic pruning and microglial phagocytosis (Barbosa et al., 2011a; Lee et al., 2015; Sochocka et al., 2017; Liu et al., 2019; Milan-Mattos et al., 2019). Recently, some studies have evaluated circulating inflammatory mediators during different phases of BD as potential biomarkers for diagnosis or treatment, and the results were promising (Bhattacharya et al., 2016; Goldsmith et al., 2016; Sigitova et al., 2017; Rowland et al., 2018). Multiple studies have demonstrated an increase in IL-6, IL-1, IL-2, TNF-α, and TNFR1 serums, during the manic and depressive phases, compared to controls and euthymic patients (Brietzke et al., 2009a,b; Barbosa et al., 2011a; Modabbernia et al., 2013; Luo et al., 2016) while IL-4 concentration was significantly lower than in controls (Kim et al., 2007). Nonetheless, other studies have found elevated IL-6 in mania and euthymia, but not in bipolar depression (Uyanik et al., 2015; Jacoby et al., 2016). Kauer-Sant'Anna et al. (2009) found that IL-6 levels were elevated during the advanced stages of disease progression, while Hamdani et al. (2012) reported that the anti-inflammatory IL-10, increased in the early stages of the disease but decreased in the final stages. It is consistent with chronic progressive neuroinflammation, where BD patients with a more significant number of previous episodes, exhibit higher levels of TNF-α and IL-6 during all disease states (Kauer-Sant'Anna et al., 2009).

Other changes in these biomarkers have been observed during different phases following the treatment of acute illness. For instance, levels of the endogenous interleukin receptor antagonist (IL-1RA) were lower in the manic stage among chronic patients, while IL-6 was higher in the euthymic phase but not during the depressive phase compared to controls. Furthermore, IL-1β levels were also significantly elevated in chronic euthymic BD (Hamdani et al., 2013; Goldsmith et al., 2016). This IL-1 activates the transcription factor NF-κB, which in turn enhances the expression and release of IL-6, IL-8, and interferon-gamma (Magalhães et al., 2012; Kany et al., 2019) Thus, Rowland et al. (2018) concluded that while no single biomarker was able to differentiate mood phases or evaluate the stages of the disease, specific combinations including IL-6, BDNF, TNF-α, TNFR1, IL-2, IL-10, and IL-4, were correlated with the disease's stages. These findings demonstrate a significant link between the immune system and BD pathophysiological pathways.

TCA Cycle Enzymes and Metabolites

TCA cycle, a crucial component of respiratory metabolism, is composed of a set of eight enzymes present in the mitochondrial matrix. However, most of the TCA cycle enzymes are encoded in the nucleus in higher eukaryotes (Cavalcanti et al., 2014). Studies suggest that mitochondrial dysfunctions play an essential role in the pathophysiology of BD. Increased neuronal oxidative stress produces deleterious effects on signal transduction, plasticity, and cell resilience (Olmez and Ozyurt, 2012), which can induce mitochondrial dysfunctions reducing the ETC activity and of the TCA cycle. The impact of these dysfunctions can be measured in the peripheral blood and postmortem brains of BD patients (De Sousa et al., 2014a; Valvassori et al., 2018). This notion is consistent with parallel transcriptomics, proteomics, and metabolomics studies, showing differential expression of numerous genes related to mitochondrial functions and oxidative stress between patients with mental disorders and healthy controls (Prabakaran et al., 2004). For instance, a loss of function mutation in the malic dehydrogenase enzyme gene, which converts malic acid to pyruvate, was found in the postmortem brains of patients with longer mental disease duration (Lee et al., 2007).

Changes in the TCA cycle, modify brain metabolism, and produce free radicals, leading to further dysfunction. The final product of glycolysis, pyruvate, is converted to acetyl-CoA by pyruvate dehydrogenase. Pyruvate, the end-product of glycolysis, is derived from cellular cytoplasm being destined into mitochondria as fuel undergirding the TCA carbon flux, being critical for mitochondrial ATP generation and for driving several major biosynthetic pathways in TCA (Gray et al., 2014). Acetyl-CoA is then converted to CO_2 in the TCA cycle with the resulting production of NADH and $FADH_2$, the electron donors for the ETC, and ATP production. Eight enzymes control the TCA cycle and inactivating anyone can reduce mitochondrial energy generation (Blass and Brown, 2000; Shi and Tu, 2015; Lazzarino et al., 2019). Despite its importance, few studies have evaluated the activity of the TCA cycle enzymes in patients with mental diseases. Bubber et al. (2011) demonstrated that the activities of enzymes in the TCA cycle varied considerably in the human brain in schizophrenia. They determined, on the prefrontal cortex, the activities of the PDHC, aconitase, isocitrate dehydrogenase (ICDH), and KGDHC. The activity of aconitase was undetectable, and the KGDHC and ICDH activities were very low. On the other hand, fumarase and malate dehydrogenase had the highest activity, while pyruvate dehydrogenase complex (PDHC) and citrate synthase activities were intermediate. Reduced activity of some of these enzymes suggests that some patients with schizophrenia have abnormalities in neural mitochondria. Interestingly, Bubber et al. (2004) had demonstrated that the enzymatic activities of the TCA cycle in mouse brains have a similar pattern, although the majority of the enzyme activities in the brain were 2–3 times higher than in humans brains. However, a study involving 18 untreated BD patients in major depressive episodes found no changes in TCA cycle enzymes compared to controls. The research assayed the activity of the key TCA cycle enzymes citrate synthase, malate dehydrogenase, and succinate dehydrogenase from leukocytes of BD patients (de Sousa et al., 2015). In contrast, Yoshimi et al. (2016) showed that serum levels of pyruvate and α-ketoglutarate in BD patients were significantly higher than those of healthy controls, while serum levels of acetyl-CoA and oxaloacetate were not altered. It is possible that TCA cycle enzymatic alterations are present only during the manic phases. Further, no study has been conducted during later disease stages. Although the BD patients presented higher serum levels of α-ketoglutarate and pyruvate than controls, the reasons underlying are unknown.

Increased pyruvate levels likely play a role in the pathogenesis of BD. The α-ketoglutarate is a key metabolite in the TCA, but also an obligatory substrate for 2-oxoglutarate-dependent dioxygenases (2-OGDO) which are involved in DNA and histone methylation, producing an epigenetic impact (Salminen et al., 2014). Altered α-ketoglutarate levels in BD may lead to epigenetic changes. Epigenetic modifications have been suggested to play an important role in the pathogenesis of many psychiatric disorders including BD (Labrie et al., 2012; Kato and Iwamoto, 2014).

Findings from animal model studies also implicate dysregulation of the TCA cycle in mental illness. Valvassori et al. (2014) found reduced levels of the TCA cycle enzyme citrate synthase, succinate dehydrogenase, and malate dehydrogenase in the prefrontal cortex, hippocampus, and striatum in amphetamine-treated rats, a commonly employed animal model of mania. Further, these reduced levels were associated with behavioral hyperactivity.

Oxidative Stress Markers

The role of oxidative stress in BD pathophysiology has been investigated in several studies. Increased neuronal oxidative stress produces deleterious effects on signal transduction, plasticity, and cell resilience (Olmez and Ozyurt, 2012), which can induce mitochondrial dysfunctions and reduce ETC activity that can be measured in the peripheral blood and postmortem brains of BD patients (De Sousa et al., 2014b; Valvassori et al., 2018). BD is characterized by alterations in CAT, SOD, and GSH-Px activity, NO, and GSH levels, DNA damage, and lipid peroxidation (Gawryluk et al., 2011; Tunçel et al., 2015). Although there is a great deal of work demonstrating serum changes in antioxidant enzymes across all BD stages, these findings are conflicting. Because of these discrepant results, these enzymes cannot yet be used as BD biomarkers, but may nonetheless be useful for evaluating disease stages (Gama et al., 2013).

As related above, studies in animals and humans have reported that increased oxidative damage reduced BDNF expression. However, BDNF has been shown to stimulate the expression and activities of GSH-Px and SOD; resulting in reduced oxidative damage (He and Katusic, 2012; Valvassori et al., 2015; Wang et al., 2020).

Clinical studies in individuals with schizophrenia or BD, provide an empirical basis for hypothesizing that abnormal BDNF and oxidative stress regulation observed in these disorders are inter-related (Fernandes et al., 2011; Zhang et al., 2015; Mansur et al., 2016). However, two recent studies reported that patients with BD exhibited a negative association between serum BDNF levels and lipid peroxidation (Tsai and Huang, 2015; Newton et al., 2017). Also, studies in schizophrenic populations observed a positive correlation between BDNF and TBARS, and a negative association between BDNF and SOD activity (Gama et al., 2008; Zhang et al., 2015). Nonetheless, there is significant heterogeneity in results within and across studies of these systems, which limits the generalizability of the findings. For example, metabolic comorbidities, obesity, and impaired glucose metabolism affect the activities of BDNF and antioxidant enzymes (Tinahones et al., 2009). Studies have demonstrated increased serum SOD activity in patients during manic or depressive episodes (Kunz et al., 2008). Increased SOD activity has been reported in medicated or unmedicated patients during manic episodes (Salim et al., 2011) and acute BD episodes, but not in euthymic patients (Singh et al., 2010). One study found CAT increases in euthymic and manic patients regardless of medication status (Steckert et al., 2010). In contrast, another found that CAT was decreased in euthymic patients but increased in unmedicated manic patients (Raffa et al., 2012). Halliwell (2006) reported increased SOD, CAT, and GSH-Px in patients during manic and depressive BD episodes.

Conversely, Vasconcelos-Moreno et al. (2017) found that GSH-Px activity was reduced in euthymic BD I patients compared to controls. A meta-analysis that included 27 studies, with 971 patients, measured eight peripheral oxidative stress markers in BD. Markers of lipid peroxidation, DNA/RNA damage, and NO were significantly increased in all stages in BD I/II patients compared to healthy controls (Brown et al., 2014; Scola et al., 2016). Andreazza et al. (2010) reported higher levels of mitochondrial protein oxidation in patients with BD, whereas another meta-analysis concluded that TBA-RS levels might be higher during manic or depressive episodes than during remission. Additionally, NO concentrations were elevated in BD patients regardless of mood state (Savas et al., 2006; Siwek et al., 2016).

CONCLUSION

Clinical and animal studies have identified multiple promising BD biomarkers that may be related to neuroinflammation, and that may alter its concentrations throughout mood episodes, showing that patients can present increased systemic toxicity during manic and depressive episodes, compared to euthymic patients (Kapczinski et al., 2010, 2011). Despite the systemic toxicity and consequent allostatic overload associated with BD, a causal link between these characteristics has yet to be established. However, the co-occurrence of acute BD episodes, clinical comorbidities, and substance abuse indicates that initial allostatic loading can produce a long-term overload effect. This state of chronic systemic toxicity occurs mainly by the dysregulation of cytokine signaling and the consequent mitochondrial oxidative stress, producing neuroinflammation, which leads to decreased BDNF expression. This observation supports the neuroprogression hypothesis and may explain, at least partially, the deficits associated with chronic BD. However, the mechanisms contributing to lower BDNF are not yet fully understood. It has been suggested that the methylation of BDNF gene promoters, can epigenetically modulate BDNF transcription and that mitochondrial oxidative stress and cytokine levels may alter the binding of nuclear transcription factors (Martinowich et al., 2003). Also, as related above, the brain is particularly susceptible to oxidative damage due to its high rate of oxygen use, lipid constitution, and low antioxidant defenses. In BD, the prevailing hypothesis is that a fundamental disturbance in mitochondrial functions generates a higher oxidative stress load. Changes in Complexes I, II, and III paired with reduced GSH levels have been

detected in BD (Andreazza et al., 2010). Further, significant increases in SOD, CAT, and GSH-Px activities have been found, suggesting the induction of compensatory mechanisms to counter the pro-oxidative state. NO levels and oxidative damage to lipids have also been identified as potential systemic toxicity markers in BD patients (Andreazza et al., 2009). These findings support the vital contribution of oxidative stress to BD neuroinflammation, and the clinical neuroprogression, justifying the research on antioxidant mechanisms as new therapeutic strategies (Pandya et al., 2013). Thus, it is hypothesized that the treatment with stabilizers drugs during the early stages of BD, may be beneficial by offering neuroprotection and slowing systemic toxicity progression through the increasing of BDNF. Also, this toxicity may be linked to age and the number of episodes. With each episode, lower BDNF levels result in more significant cognitive impairment and reduced functionality, further reducing the chances of returning to euthymia. Indeed the number of episodes has a more significant impact on disease evolution, than the patient's age (Passos et al., 2016; Scussel et al., 2016).

Thus, based on our improved understanding of the neuroinflammation and neuroprogression, it is reasonable to speculate that combinations of biomarkers for different pathophysiological processes of BD, will 1 day help predict disease evolution, treatment response, and long-term outcomes (Goldberg and Harrow, 2004). However, the interactions among biomarkers are complex and, as of yet, cannot predict an outcome. Thus, as questioned by Vinberg (2020), in a recent article, the search for peripheral biomarkers in psychiatry is like searching for the needles in a haystack.

Thereby, this review sought to clarify some pathophysiological mechanisms of BD, focusing mainly on the energetic metabolism of brain cells and their correlation with mental diseases.

AUTHOR CONTRIBUTIONS

LC and DD: conception of the idea, drafting and revising the article and revising the article and correspondence. LC, OU, and TM: bibliographic research and main drafting. OU: elaboration of figures. All authors contributed to the article and approved the submitted version.

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Nitric Oxide in the Spinal Cord Is Involved in the Hyperalgesia Induced by Tetrahydrobiopterin in Chronic Restraint Stress Rats

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It has been well recognized that exposure to chronic stress could increase pain responding and exacerbate pain symptoms, resulting in stress-induced hyperalgesia. However, the mechanisms underlying stress-induced hyperalgesia are not yet fully elucidated. To this end, we observed that restraint as a stressful event exacerbated mechanical and thermal hyperalgesia, accompanied with up-regulation of nitric oxide (NO) ($P < 0.001$), GTP cyclohydrolase 1 (GCH1) (GCH1 mRNA: $P = 0.001$; GCH1 protein: $P = 0.001$), and tetrahydrobiopterin (BH4) concentration (plasma BH4: $P < 0.001$; spinal BH4: $P < 0.001$) on Day 7 in restraint stress (RS) rats. Intrathecal injection of N^{ω}-nitro-L-arginine methyl ester (L-NAME), a non-specific NO synthase inhibitor, or *N*-([3-(aminomethyl)phenyl]methyl) ethanimidamide, a special inhibitor of inducible NO synthase (iNOS), for seven consecutive days attenuated stress-induced hyperalgesia and decreased the production of NO ($P < 0.001$). Interestingly, 7-nitro indazole, a special inhibitor of neuronal NO synthase, alleviated stress-induced hyperalgesia but did not affect spinal NO synthesis. Furthermore, intrathecal injection of BH4 not only aggravated stress-induced hyperalgesia but also up-regulated the expression of spinal iNOS (iNOS mRNA: $P = 0.015$; iNOS protein: $P < 0.001$) and NO production ($P < 0.001$). These findings suggest that hyperalgesia induced by RS is associated with the modulation of the GCH1–BH4 system and constitutively expressed spinal iNOS. Thus, the GCH1–BH4–iNOS signaling pathway may be a new novel therapeutic target for pain relief in the spinal cord.

Keywords: tetrahydrobiopterin, stress-induced hyperalgesia, spinal cord, GTP cyclohydrolase 1, inducible nitric oxide synthase

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INTRODUCTION

Moderate stress within the range of physiological adaptation could exert beneficial effects on pain alleviation, which has currently been recognized as stress-induced analgesia (SIH). On the contrary, it should be noted that stress also has detrimental actions for pain sensors via eliciting a down-regulation of pain sensitivity, causing the onset of SIH by a wide variety of stress factors, including

repeated exposure to the cold environment, restraint, and forced swimming (Satoh et al., 1992; Quintero et al., 2000; da Silva Torres et al., 2003; Imbe et al., 2004, 2010, 2012). Additionally, stress has detrimental effects on several physiological functions, such as affecting behavioral and physiological homeostasis and disturbing neurogenesis, promoting vulnerable susceptibility, and ultimately aggravating damage to neurological systems (Selye, 1976; Kim and Yoon, 1998; McEwen, 1998; Selye, 1998). Several mechanisms have been demonstrated to explain the hyperalgesia induced by chronic stress, including opioid, gamma-aminobutyric acid (GABA), glutamate, monoamine, endocannabinoid, sympathetic adrenomedullary systems, and the hypothalamic–pituitary–adrenal (HPA) axis (Jennings et al., 2014). For instance, the decrease of GABA release and GABA-receptor activation in the spinal cord involves forced swimming SIH and pain-induced c-Fos overexpression (Suarez-Roca et al., 2008; Quintero et al., 2011; Ma et al., 2014). The switch of endogenous opioid signaling from an antinociceptive to a pronociceptive pathway involves chronic SIH (Ferdousi and Finn, 2018). Furthermore, a contribution of the activation of the HPA axis and sympathetic nervous system to SIH has been demonstrated (Elenkov et al., 2000; Golovatscka et al., 2012). Nevertheless, the mechanism underlying SIH has not been fully elucidated.

Tetrahydrobiopterin (BH4) is an essential cofactor for three isoforms of nitric oxide (NO) synthase (NOS): neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). It plays a crucial role in regulating NOS and biosynthesis of NO (Thony et al., 2000; Tegeder et al., 2006; Lam et al., 2007; Werner et al., 2011). Currently, accumulating evidence shows that BH4 is likely to be involved in exacerbating neuropathic and inflammatory pain (Tegeder et al., 2006; Latremoliere et al., 2015), resulting in increased pain sensitivity. Additionally, intrathecal infusion of BH4 could induce and exacerbate nociception by facilitating central sensitization (Nasser et al., 2015). Reducing BH4 production and availability may facilitate the physiological benefits for hyperalgesia in humans. Previous research suggested that BH4 was involved in the pain signaling pathways by regulating neurotransmitters' biosynthesis, including noradrenaline, adrenaline, dopamine, serotonin, and NO (Thony et al., 2000; Kolinsky and Gross, 2004). Moreover, BH4 induces pain sensitivity partly by the regulation of excess production of NO from nNOS in the L4-5 spinal dorsal root ganglions in the spared nerve injury model of peripheral neuropathic pain (Tegeder et al., 2006), and NO might be involved in the activation of guanylyl cyclase–cGMP–PKG pathway and regulation of the activity of *N*-methyl-D-aspartate (NMDA) receptor (Lipton et al., 1993; Tegeder et al., 2004, 2006).

The biosynthesis of BH4 is highly controlled by three main pathways: the *de novo* synthetic pathway, the salvage pathway, and the cycling pathway. GTP cyclohydrolase 1 (GCH1) is the rate-limiting enzyme responsible for the *de novo* pathway of BH4 synthesis, which could catalyze the initial reaction and convert GTP to 7,8-dihydroneopterin triphosphate (Werner et al., 2011). There is evidence that GCH1 is also involved in developing neuropathic and inflammatory pain. Moreover, intraperitoneal or intrathecal injection (i.t.) of 2,4-diamino-6-hydroxypyrimidine (DAHP), the prototypical GCH1 inhibitor, has antinociceptive effect in the chronic constriction injury (CCI) models and spinal nerve ligation (SNL) models (Tegeder et al., 2006).

Given the critical role of spinal BH4 in hyperalgesia, we aimed to investigate whether SIH is associated with the GCH1–BH4–NO system. To this end, we first established the chronic restraint stress (RS) model to investigate its effects on pain intensity and clarify its relationship with spinal GCH1. We further designed to observe whether BH4 in SIH is mediated mainly by NO. Finally, the present study using a specific NOS inhibitor to validate NO comes from which NOS isoform mainly modulates pain responses. Collectively, our data for the first time implicate the role of spinal BH4 in hyperalgesia.

RESULTS

GTP Cyclohydrolase 1–Tetrahydrobiopterin Axis Was Involved in the Development and Maintenance of Stress-Induced Analgesia

Chronic Restraint Stress-Induced Time-Dependent Mechanical and Thermal Hyperalgesia

Paw withdrawal mechanical threshold (PWMT) test, paw withdrawal thermal latency (PWTL), and tail-flick latency (TFL) were commonly used to evaluate pain sensitivity (Sung et al., 2004; Guan et al., 2015; Deciga-Campos et al., 2016). All tests were conducted on the first day before establishing the RS model and on the third, fifth, and seventh days. Before the model was established, both left hind paws' mechanical pain sensitivity and thermal pain sensitivity had no statistical difference ($P > 0.05$). On the third, fifth, and seventh days of RS, the PWMT of the RS rats was significantly decreased to 6.94 ± 1.24, 6.67 ± 0.9, and 5.22 ± 0.94 g, respectively (RS effect: $F_{1,22} = 192.322$, $P < 0.001$; observation intervals: $F_{3,66} = 22.641$, $P < 0.001$; interaction: $F_{3,66} = 16.798$, $P < 0.0001$, **Figure 1A**), suggesting that RS has been successfully developed as a model of mechanical tactile allodynia. As to the PWTL, the increase in %MPE was significantly decreased in RS rats compared with that of age-matched control rats (**Figure 1B**), which represented time-dependent thermal hyperalgesia. Compared with the control rats, %Analgesia in the RS group was significantly decreased on Day 3, Day 5, and Day 7, while %Analgesia of rats subjected to RS was slightly decreased on Day 5, and Day 7 compared with Day 3 (RS effect: $F_{1,22} = 5.457$, $P = 0.029$; observation intervals: $F_{2,44} = 0.002$, $P = 0.998$; interaction: $F_{2,44} = 2.552$, $P = 0.089$, **Figure 1C**). Therefore, it is likely that the RS models and their related SIH, including abnormality in mechanical allodynia and thermal hyperalgesia, were successfully established.

Chronic Restraint Stress-Induced Hypomethylation of GTP Cyclohydrolase 1, Thus Up-Regulating the Expression of Spinal GTP Cyclohydrolase 1

DNA methylation is an essential mechanism for the control gene, mainly in the CpG islands (CGIs). We analyzed the CGI across the whole GCH1 gene (i.e., 5,000 bp upstream of the first

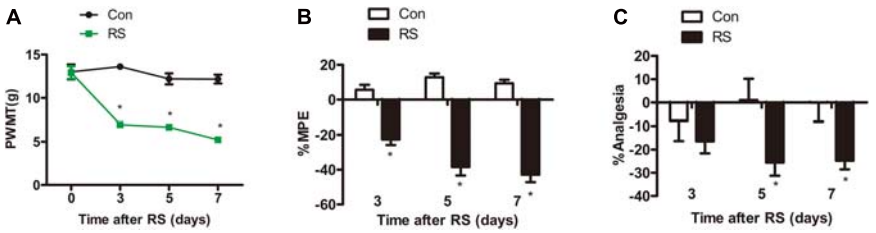

FIGURE 1 | Mechanical or thermal nociceptive thresholds in rats with or without chronic RS. **(A–C)** Nociceptive behavior tests including PWMT (RS effect: $F_{1,22} = 192.322$, $P < 0.001$; observation intervals: $F_{3,66} = 22.641$, $P < 0.001$; interaction: $F_{3,66} = 16.798$, $P < 0.001$), PWTL (RS effect: $F_{1,22} = 141.084$, $P < 0.001$; observation intervals: $F_{2,44} = 4.606$, $P = 0.015$; interaction: $F_{2,44} = 12.632$, $P < 0.001$), and TFL (RS effect: $F_{1,22} = 5.457$, $P = 0.029$; observation intervals: $F_{2,44} = 0.002$, $P = 0.998$; interaction: $F_{2,44} = 2.552$, $P = 0.089$). Data are shown as mean ± SEM ($n = 12$). *Significant difference with respect to control groups (two-way ANOVA with repeated measures in nociceptive behavior tests, followed by Bonferroni *post hoc* test or Dunnett's T3 test if necessary). *$P < 0.05$. RS, restraint stress; PWMT, paw withdrawal mechanical threshold; PWTL, paw withdrawal thermal latency; TFL, tail-flick latency; NO, nitric oxide; iNOS, inducible nitric oxide synthase.

exon to 1,000 bp downstream of the last exon) using the CGI prediction software because of no available annotation for the transcription of the start and end of the rats GCH1. Programs #21 and #22 (**Table 1**) were the recommendations, and a total of 77 CGIs were investigated. As we expected, RS rats represented a hypomethylation of GCH1 in the location of CpG 14, 15 (27909945, 27909939) and CpG 39 (27909657) compared with the control rats (CpG 14, 15: $P < 0.001$; CpG 39: $P = 0.016$, **Table 2**). Moreover, GCH1 demethylation induced the activation and expression of GCH1 mRNA and protein (GCH1 mRNA: $P = 0.001$; GCH1 protein: $P = 0.001$, **Figures 2A,B**).

Effects of Chronic Restraint Stress on the Biosynthesis of Spinal and Plasma Tetrahydrobiopterin

GTP cyclohydrolase 1 is the first rate-limiting enzyme of BH4 in the *de novo* synthesis; thus, the GCH1 gene's demethylation and activation of GCH1 mRNA and protein might improve the biosynthesis of BH4. Therefore, the plasma and spinal BH4 were detected by high-performance liquid chromatography (HPLC). As demonstrated in **Figures 2C,D**, the content of BH4 in the plasma was significantly increased to the spinal BH4 compared with the control rats (plasma BH4: $P < 0.001$; spinal BH4: $P < 0.001$).

Chronic Restraint Stress-Induced Analgesia Was Attenuated by DAHP and Exacerbated by Intrathecal Injection of Tetrahydrobiopterin Into the Spinal Cord

To further investigate the functional role of spinal GCH1 and BH4 in SIH, rats were intrathecally injected with either BH4 or DAHP 15 min prior to RS, respectively. As shown in **Figures 3A–C**, on Days 3, 5, and 7, the specific inhibitor of GCH1 DAHP (6 mg/kg, i.t.) presented an analgesic effect in RS rats by evaluation of the PWMT, PWTL, and TFT in the same hind paws compared with the vehicle-treated rats (PWMT: treatment: $F_{2,23} = 73.995$, $P < 0.001$; observation intervals: $F_{3,69} = 45.069$, $P < 0.001$; interaction: $F_{6,69} = 11.544$, $P < 0.0001$; %MPE: treatment: $F_{2,23} = 51.543$, $P < 0.001$; observation intervals: $F_{2,46} = 3.942$, $P = 0.026$; interaction: $F_{4,46} = 0.761$, $P = 0.556$; %Analgesia: treatment: $F_{2,23} = 50.134$, $P < 0.001$; observation intervals: $F_{2,46} = 6.665$, $P = 0.003$; interaction: $F_{4,46} = 0.257$, $P = 0.904$). Interestingly, BH4 caused a rapid and long-lasting increase of PWMT and PWTL in RS rats. DAHP (6 mg/kg, i.t.) failed to elicit any pharmacological effects on the mechanical or heat pain sensitivity in control rats, while BH4 (1 μg/μl, 10 μl, i.t.) evoked a hyperalgesia in control rats (data not shown). One plausible explanation for this discrepancy is that in healthy animals, the activity of sensory neurons in *de novo* pathway is lower, while the recycling and salvage pathways maintain the basal homeostatic BH4. However, after nerve injury or inflammation, the activation and expression of GCH1 are remarkably up-regulated in sensory neurons, thus inducing an overproduction of BH4 (Tegeder et al., 2006), suggesting that BH4 may possess pronociceptive properties at central sites of the somatosensory system.

Based on our findings, we concluded that the GCH1–BH4 system was involved in the occurrence and development of hyperalgesia induced by chronic RS. Moreover, inhibition of the *de novo* synthesis of BH4 by blocking GCH1 might be a new therapeutic target for chronic pain.

The Role of Spinal Nitric Oxide in the Development of Stress-Induced Analgesia Induced by Tetrahydrobiopterin

Considerable evidence has shown that NO has an important role in the peripheral and central nervous system and participates in a wide variety of physiologic and pathophysiologic processes, such as neurotoxicity and pathologic pain (Meller et al., 1992; Wong et al., 1998; Tang et al., 2007). Thus, we detected spinal NO expression in the RS and control rats to validate our hypothesis that NO might be involved in the SIH. As expected, RS up-regulated spinal NO expression compared with the control rats ($P < 0.001$, **Figure 4A**). To determine whether DAHP decreased spinal NO expression, we also detected spinal NO in the DAHP-treated and BH4-treated rats. As shown in **Figure 4B**, the expression of spinal NO in the DAHP group was robustly decreased compared with that of the vehicle RS rats, while it is slightly increased in the BH4 group ($F_{2,15} = 67.94$, $P < 0.001$).

TABLE 1 | Primer design.

Program #21	Primer	Start	Size	Tm	GC%	C's
	5′ primer	4811	24	59.60	33	5
	3′ primer	5166	25	60.47	40	4
Program #22	5′ primer	5142	25	60.47	40	4
	3′ primer	5719	24	60.17	29	8
Program #21 Product size: 356 No. of CPGs: 32 Coverage: 31						
Program #22 Product size: 578 No. of CPGs: 45 Coverage: 41						

Program #21: 5′ primer sequence: aggaagagagGGTTTAATTTGAGGGTTGTTTTGT; 3′ primer sequence: cagtaatacgactcactatagggagaaggctAAATAAAAAAATCCCACATTCCCT. Program #22: 5′ primer sequence: aggaagagagGGGTGTAAGGTGTATTAATGGGTTT; 3′ primer sequence: cagtaatacgactcactatagggagaaggct AAATAAAAAAATCCCACATTCCCT.

TABLE 2 | GCH1 DNA methylation values for each CpG unit in control and RS groups.

CpG sites	Position	Con (n = 6)	RS (n = 6)	P-value
CpG 14, 15	27909945; 27909939	0.2233 ± 0.04082	0.04457 ± 0.01820	**0.001
CpG 39	27909657	0.4783 ± 0.14932	0.2350 ± 0.14349	*0.016

All data are presented as the mean ± SEM. P-values were calculated with Student's test, and P < 0.05 was considered statistically significant.
**P < 0.05.*
***P < 0.001.*
RS, restraint stress.

Based on our findings, we supposed that the effect of BH4 in SIH was regulated by spinal NO. Therefore, a non-specific inhibitor of NO, N^{ω}-nitro-L-arginine methyl ester (L-NAME), was intrathecally injected into RS rats to test our hypothesis. Both L-NAME (30 μg/μl, 10 μl, i.t.) and BH4 (1 μg/μl, 10 μl, i.t.) were intrathecally injected into the RS rats, and L-NAME was administrated 30 min before BH4. Low pain sensitivity was induced in rats treated with either L-NAME/BH4 or L-NAME as compared with the vehicle-treated rats and BH4-treated rat (PWMT: treatment: $F_{3,33}$ = 47.94, P < 0.001; observation intervals: $F_{3,99}$ = 49.132, P < 0.001; interaction: $F_{9,99}$ = 16.453, P < 0.001; %MPE: treatment: $F_{3,33}$ = 34.594, P < 0.001; observation intervals: $F_{2,66}$ = 1.131, P = 0.329; interaction: $F_{6,66}$ = 3.15, P = 0.009; %Analgesia: treatment: $F_{3,33}$ = 22.943, P < 0.001; observation intervals: $F_{2,66}$ = 1.598, P = 0.21; interaction: $F_{6,66}$ = 2.904, P = 0.014, **Figures 4C–E**). Furthermore, spinal NO expression was significantly decreased in the L-NAME/BH4 group and L-NAME group compared with the vehicle group ($F_{3,20}$ = 51.906, P < 0.001, **Figure 4F**). Interestingly, BH4 intrathecally injected rats only presented a slight increase of spinal NO. This might be explained by the fact that RS induced a marked biosynthesis of BH4, and endogenous BH4 catalyzed NOS to produce NO, reaching the peak.

Inducible Nitric Oxide Synthase–Nitric Oxide Cascade System Modulated the Role of Tetrahydrobiopterin in the Stress-Induced Analgesia in the Spinal Cord

To further investigate which isoform NOS mainly modulates the role of BH4 in the SIH in the spinal cord, nNOS, iNOS, and eNOS were subsequently examined. The expression level of spinal iNOS in the RS group was remarkably up-regulated as compared with the control group rats (iNOS mRNA: P = 0.045, iNOS protein: P = 0.046, **Figures 5A,B**), while no significant differences of spinal nNOS and eNOS were observed between the RS group and control group (nNOS mRNA: P = 0.3; nNOS protein: P = 0.565; eNOS mRNA: P = 0.937; eNOS protein: P = 0.449, **Figures 5C–F**). Furthermore, the expression of spinal iNOS was remarkably up-regulated in the rat models of RS treated with BH4; however, it was distinctively down-regulated by DAHP (iNOS mRNA: $F_{2,9}$ = 6.869, P = 0.015; iNOS protein: $F_{2,9}$ = 123.036, P < 0.001, **Figures 6A,B**), which was consistent with previous behavioral results. On the other hand, compared with those in the vehicle group, spinal nNOS and eNOS were neither down-regulated in the DAHP-treated group nor up-regulated in the BH4-treated group in the rat model of RS (nNOS mRNA: $F_{2,9}$ = 0.223, P = 0.804; nNOS protein: $F_{2,9}$ = 0.131, P = 0.879; eNOS mRNA: $F_{2,9}$ = 0.879, P = 0.448; eNOS protein: $F_{2,9}$ = 0.081, P = 0.923, **Figures 6C–F**). Based on our findings, we might conclude that NO comes from the iNOS isoform mainly modulating the role of BH4 in the SIH in the spinal cord. To further validate our hypothesis, the specific inhibitor of nNOS [7-nitro indazole (7-NI), 40 μg/μl, 10 μl, i.t.] and iNOS (1400W, 1 μg/μl, 10 μl, i.t.) was subsequently administrated. As we expected, 1400 W effectively alleviated the SIH by inhibiting the iNOS–NO cascade system in the spinal cord. Interestingly, 7-NI attenuated the SIH, while it had no statistical effect on the spinal NO (PWMT: treatment: $F_{2,25}$ = 20.961, P < 0.001; observation intervals: $F_{3,75}$ = 4.045, P = 0.016; interaction: $F_{6,75}$ = 9.247, P < 0.001; %MPE: treatment: $F_{2,25}$ = 15.561, P < 0.001; observation intervals: $F_{2,50}$ = 2.038, P = 0.154; interaction: $F_{4,50}$ = 1.831, P = 0.158; %Analgesia: treatment: $F_{2,25}$ = 4.898, P = 0.016; observation intervals: $F_{2,50}$ = 3.174, P = 0.064; interaction: $F_{4,50}$ = 1.1, P = 0.362; NO: $F_{2,15}$ = 27.36, P < 0.001, **Figures 7A–D**). These findings indicate that 1400 W through inhibiting the iNOS–NO cascade system to mainly

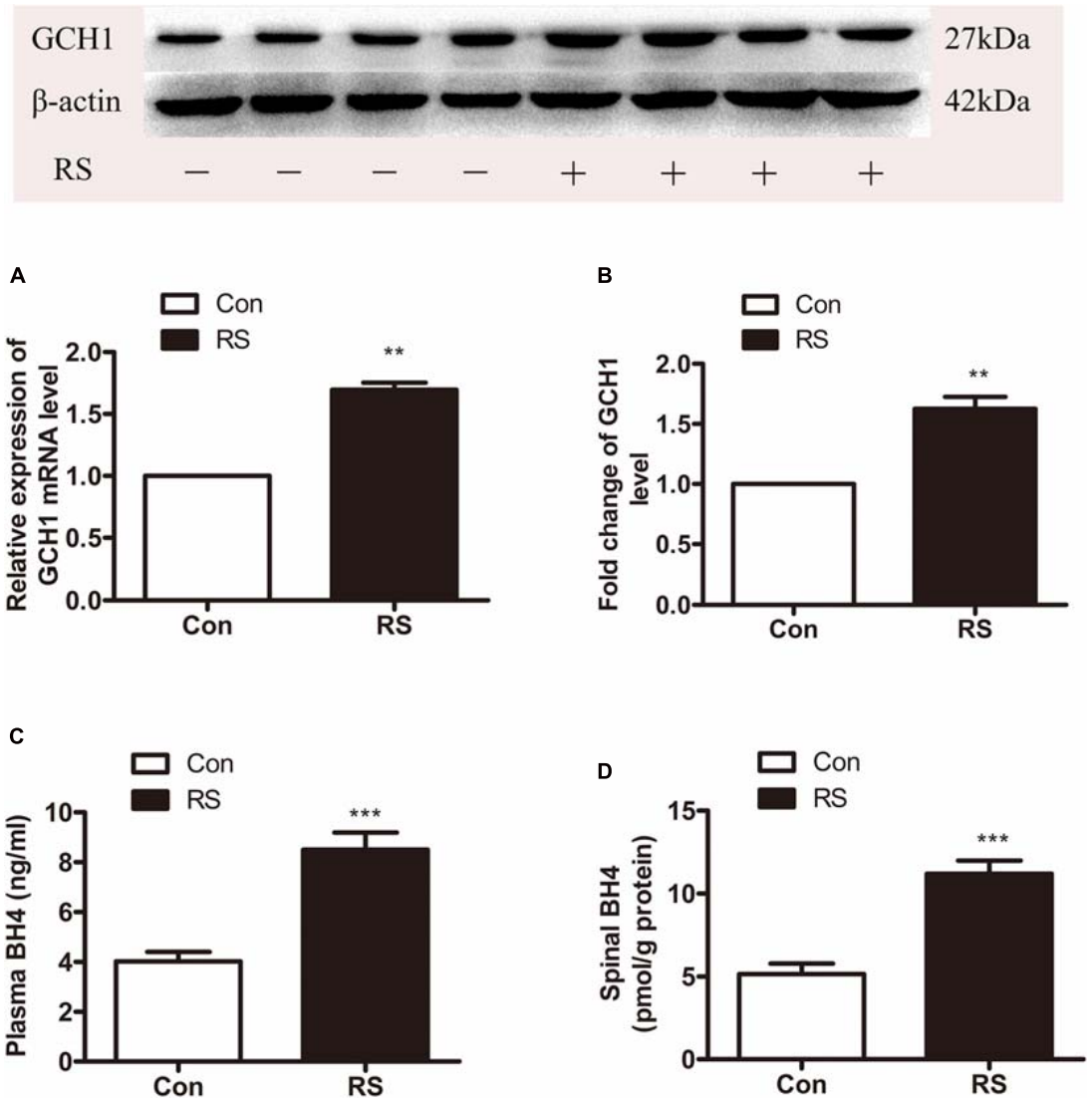

FIGURE 2 | The expression of spinal GCH1 and the production of plasma and spinal BH4 in the rats with or without chronic RS. **(A,B)** The expression of spinal GCH1 mRNA ($P = 0.001$) and spinal GCH1 protein ($P = 0.001$) in the RS rats and control rats. **(C)** The concentration of plasma BH4 ($P < 0.001$) and spinal BH4 ($P < 0.001$) in the RS and control rats. *Significant difference with respect to control groups (Student's t test). **$P < 0.01$ and ***$P < 0.001$. GCH1, GTP cyclohydrolase 1; BH4, tetrahydrobiopterin; RS, restraint stress.

modulate the role of BH4 in the SIH in the spinal cord. Furthermore, results of iNOS-immunoreactive intensity were consistent with the results of western blot and qRT-PCR analysis (**Figure 7E**). To the best of our knowledge, there are no other studies reporting on the cellular localization of iNOS in the spinal cord in RS rats. Therefore, double immunofluorescence of iNOS was performed with different cell markers, including GFAP (astrocyte biomarker), Iba1 (microglia biomarker), and NeuN (neuron biomarker). Spinal samples acquired from the RS rats indicated that iNOS was co-expressed with astrocytes in the superficial layer of the dorsal horn of the spinal cord, instead of microglia and neurons. These results indicate that iNOS induced by chronic RS in the spinal cord is produced by astrocytes (**Figure 7F**).

DISCUSSION

Chronic stress could enhance pain sensitivity and induce hyperalgesia through the supraspinal pain conduction pathway [including the cerebral cortex, amygdala, periaqueductal gray, and rostral ventromedial medulla (RVM)] and spinal dorsal horn (Jennings et al., 2014; Wippert and Wiebking, 2018). Nociceptive stimulation is transmitted to the somatosensory cortex through the ascending pain pathway. Subsequently, descending facilitatory or inhibitory pathways are considered to be activated to enhance or restrain nociceptive transmission, respectively. The descending projections of RVM to the spinal dorsal horn play a key role in SIH (Jennings et al., 2014). Although the mechanism in the spinal cord involved in the SIH

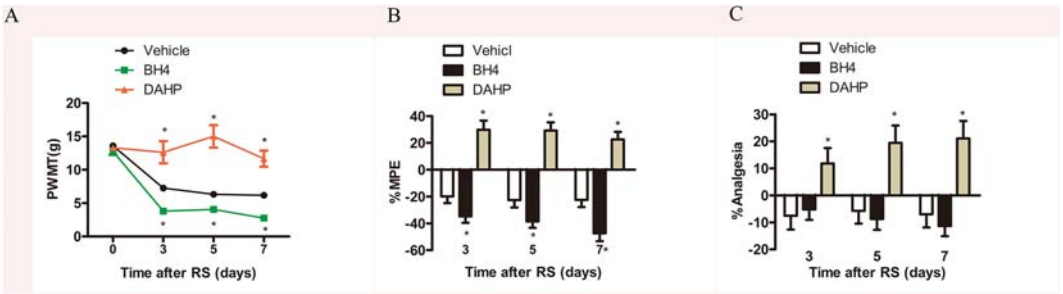

FIGURE 3 | Effects of either DAHP or BH4 intrathecal administration on nociceptive behavior in rats subjected to chronic RS. Chronic RS rats were consecutively administered with either DAHP (6 mg/kg) or BH4 (1 μg/ml, 10 μl) as the study protocol in **Figure 1**. **(A–C)** Nociceptive behavior tests including PWMT (treatment: $F_{2,23} = 73.995$, $P < 0.001$; observation intervals: $F_{3,69} = 45.069$, $P < 0.001$; interaction: $F_{6,69} = 11.544$, $P < 0.0001$); **(B)** treatment: $F_{2,23} = 51.543$, $P < 0.001$; observation intervals: $F_{2,46} = 3.942$, $P = 0.026$; interaction: $F_{4,46} = 0.761$, $P = 0.556$; **(C)** treatment: $F_{2,23} = 50.134$, $P < 0.001$; observation intervals: $F_{2,46} = 6.665$, $P = 0.003$; interaction: $F_{4,46} = 0.257$, $P = 0.904$. Data are shown as mean ± SEM (n = 8–10). *Significant difference with respect to vehicle groups (two-way ANOVA with repeated measures in nociceptive behavior tests, followed by Bonferroni *post hoc* test or Dunnett's T3 test if necessary, $^*P < 0.05$). BH4, tetrahydrobiopterin; RS, restraint stress; PWMT, paw withdrawal mechanical threshold.

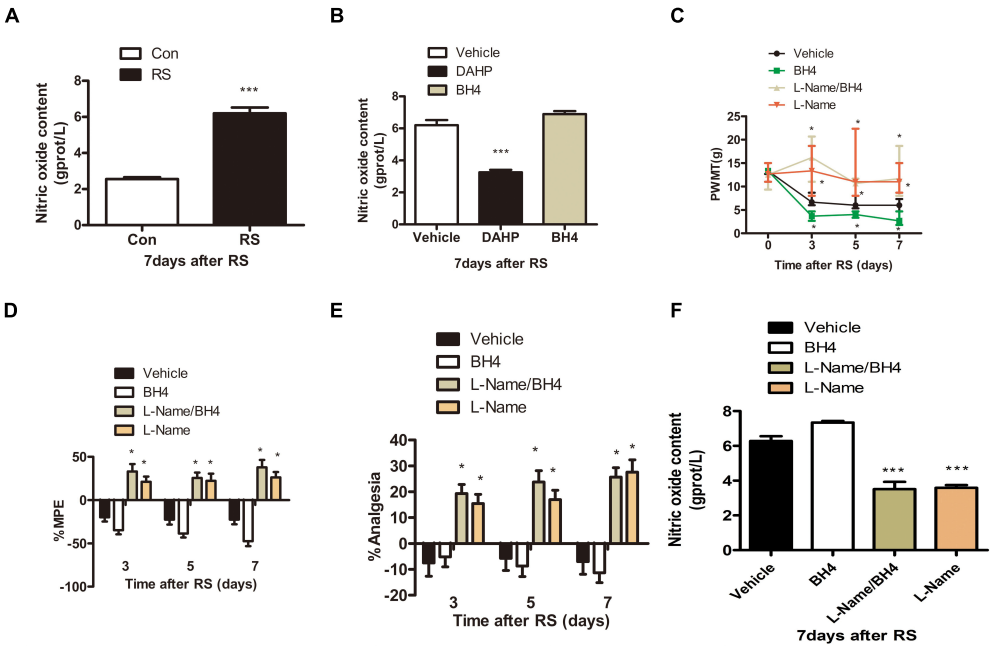

FIGURE 4 | The expression of spinal NO in the rats subjected or not subjected to chronic RS and effect of L-NAME on the nociceptive behavior and spinal NO in the rat models of RS. **(A)** The expression of spinal NO in the rats subjected or not subjected to chronic RS, $P < 0.001$. **(B)** Effects of either DAHP or BH4 intrathecal administration on spinal NO in rats subjected to chronic RS. Chronic RS rats received chronic intrathecal treatment with either DAHP (6 mg/kg) or BH4 (1 μg/ml, 10 μl) from the first day of establishing chronic RS until the end of the experiment, $F_{2,15} = 67.94$, $P < 0.001$. **(C–E)** Effect of L-NAME intrathecal administration on nociceptive behavior in rats subjected to chronic RS; chronic RS rats that received chronic intrathecal treatment with L-NAME (30 μg/μl, 10 μl) 30 min prior to BH4 (1 μg/ml, 10 μl), L-NAME (30 μg/μl, 10 μl), and BH4 (1 μg/ml, 10 μl) treatment. Nociceptive behavior tests including PWMT (treatment: $F_{3,33} = 47.94$, $P < 0.001$; observation intervals: $F_{3,99} = 49.132$, $P < 0.001$; interaction: $F_{9,99} = 16.453$, $P < 0.0001$), PWTL (treatment: $F_{3,33} = 34.594$, $P < 0.001$, observation intervals: $F_{2,66} = 1.131$, $P = 0.329$; interaction: $F_{6,66} = 3.15$, $P = 0.009$), and TFL (treatment: $F_{3,33} = 22.943$, $P < 0.001$; observation intervals: $F_{2,66} = 1.598$, $P = 0.21$; interaction: $F_{6,66} = 2.904$, $P = 0.014$). **(F)** Effect of intrathecal administration of L-NAME on the expression of spinal NO, $F_{3,20} = 51.906$, $P < 0.001$. *Significant difference with respect to control or vehicle groups (two-way ANOVA with repeated measures). $^*P < 0.05$ and $^{***}P < 0.001$. NO, nitric oxide; RS, restraint stress; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NO, nitric oxide; BH4, tetrahydrobiopterin; PWMT, paw withdrawal mechanical threshold; PWTL, paw withdrawal thermal latency; TFL, tail-flick latency.

remains elusive, previous studies have demonstrated that the occurrence and maintenance of different pain share a common signal transduction pathway of BH4 in various pain models (Tegeder et al., 2006; Kim et al., 2009; Latremoliere and Costigan, 2011). Nevertheless, there is no evidence showing that no injury stress-induced hyperalgesia is dependent on BH4. In the present study, our results demonstrated that (i) chronic RS could induce mechanical allodynia and thermal hyperalgesia in the rat model;

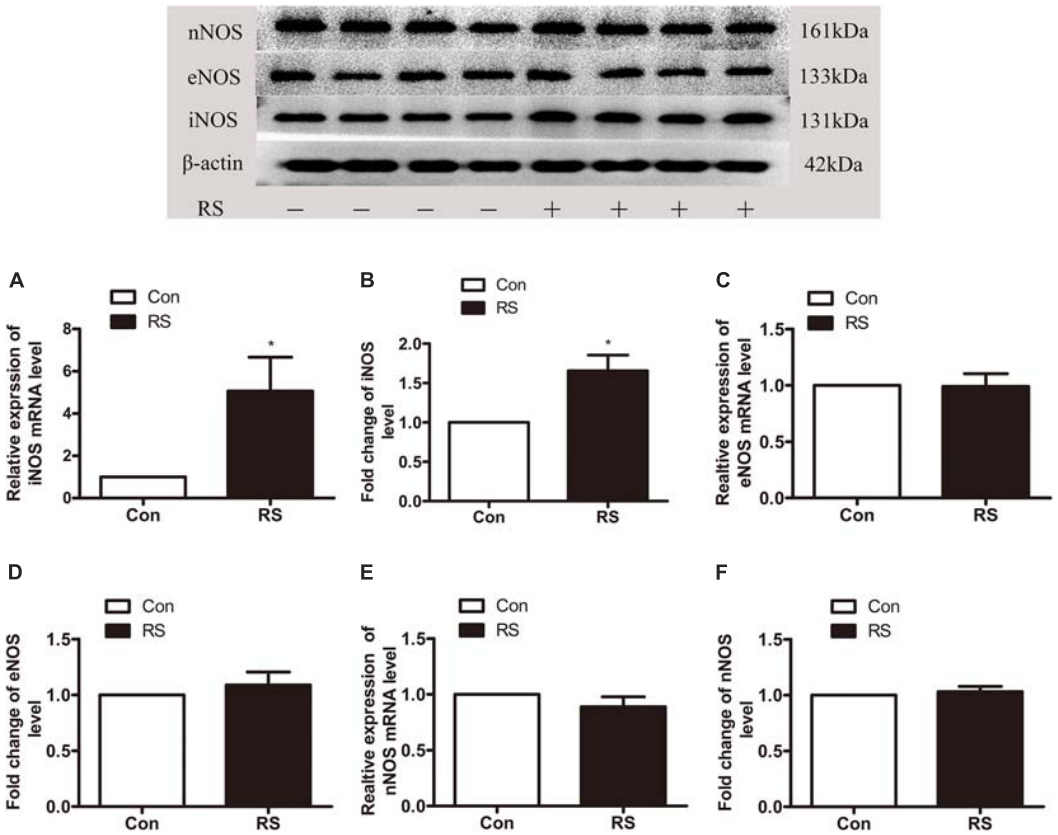

FIGURE 5 | The expression of three isoforms of NOS in the spinal cord in the chronic RS or control rats. **(A,B)** The expression of spinal iNOS mRNA ($P = 0.045$) and protein ($P = 0.046$) in the rats subjected or not subjected to chronic RS. **(C,D)** The expression of spinal eNOS mRNA ($P = 0.937$) and protein ($P = 0.449$) in the rats subjected or not subjected to chronic RS. **(E,F)** The expression of spinal nNOS mRNA ($P = 0.3$) and protein ($P = 0.565$) in the RS rats or control rats. Data are presented as mean ± SEM ($n = 4$), $^*P < 0.05$. NOS, nitric oxide synthase; RS, restraint stress; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase.

(ii) the gene of GCH1 demethylation was concomitant with the up-regulation of spinal GCH1 mRNA and protein in the chronic RS rats; (iii) chronic RS-induced hyperalgesia might be partly regulated by the overproduction of NO, accompanied with BH4; and (iv) the activation of iNOS–NO cascade system in the spinal cord partly modulated the SIH. In general, our study first implicates a critical role of BH4 in the occurrence and development of SIH, but the precise mechanisms are not clear.

GTP cyclohydrolase 1, the first rate-limiting enzyme in the *de novo* synthesis of BH4, has been implicated in developing and maintaining neuropathic and inflammatory pain (Nasser and Moller, 2014). Specifically, we found an up-regulation of GCH1 mRNA, accompanied by increased protein expression in the chronic RS on 7 days. In support of this, demethylation of gene GCH1 elicited by repeated exposure to RS was observed. This study is the first to validate the correlation between SIH development and GCH1 methylation in preclinical studies. Accumulating evidence has demonstrated that DNA methylation is an essential modification of protein and nucleic acid. It could modulate the expression and closure of genes closely associated with many diseases (e.g., cancer). Thus, it is a critical component of epigenetic machinery (Wang et al., 2016). Tegeder et al. (2006) have declared that single-nucleotide polymorphisms (SNPs) in the gene for the GCH1 alter responses to noxious stimuli in healthy humans and susceptibility to neuropathic pain in patients. Nevertheless, the precise locations mediating the regulation of GCH1 transcription are not determined in their study. For the first time, our study elucidates that three CGIs (27909945, 27909939, and 27909657) regulate it, providing a function gene therapy for chronic pain. To determine if blocking GCH1 attenuates pain sensitivity, we intrathecally administrated GCH1 unique inhibitor DAHP (6 mg/kg) via a lumbar spinal catheter. As expected, DAHP effectively attenuated mechanical allodynia and thermal hyperalgesia in rats exposed to RS. Interestingly, unlike stress-elicited pain conditions, DAHP did not influence behavioral responses to stimuli, consistent with the previous study (Tegeder et al., 2006). A previous observation has demonstrated that DAHP-mediated inhibition of GCH1 may be GFRP-dependent, and DAHP could selectively inhibit GCH1 activity via competition for substrate GTP (Kolinsky and Gross, 2004). However, in the prevention study, the precise mechanisms of DAHP inhibition GCH1 required clarity in further studies. Furthermore, it has been established that GCH1 activity is subjected to feedback inhibition by the *de novo*

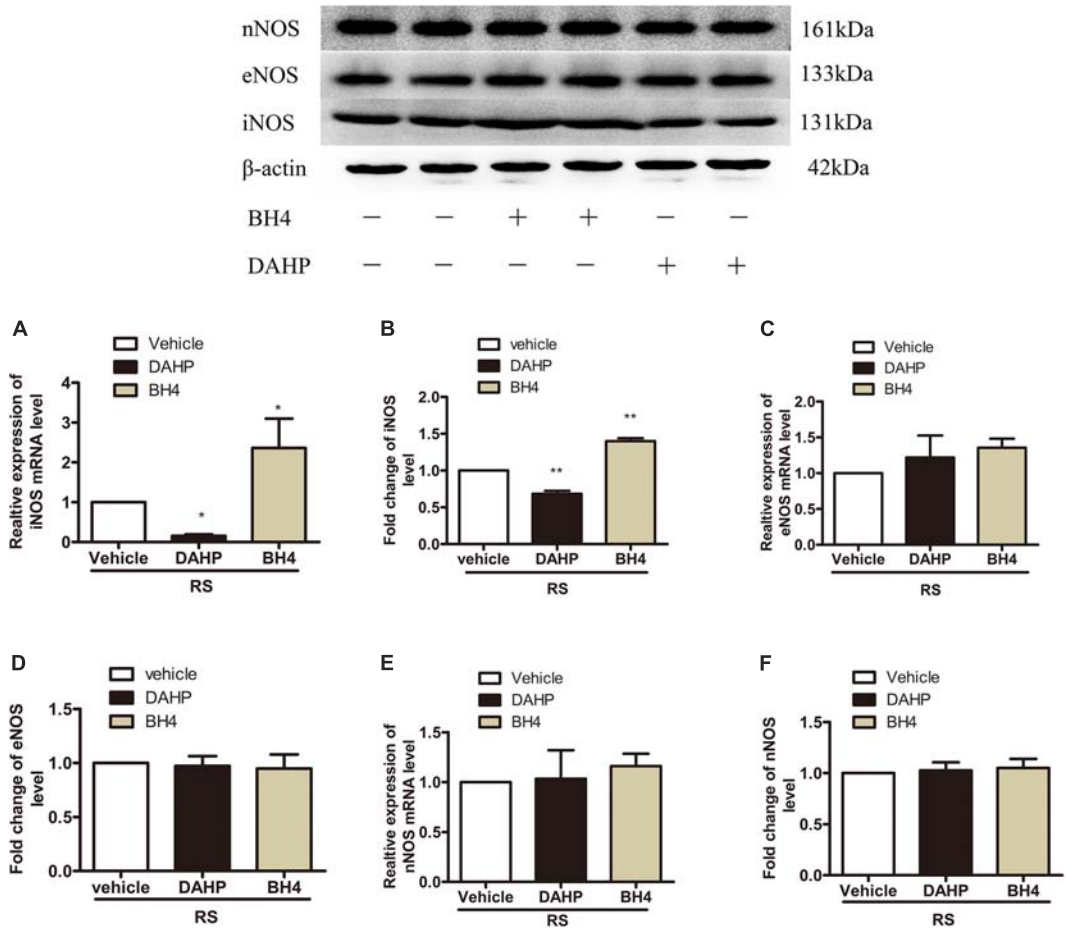

FIGURE 6 | Effect of either DAHP or BH4 intrathecal administration on the expression of three spinal NOS isoforms. **(A,B)** The expression of spinal iNOS mRNA ($F_{2,9}$ = 6.869, P = 0.015) and protein ($F_{2,9}$ = 123.036, $P < 0.001$) in rats treated with DAHP or BH4. **(C,D)** The expression of spinal eNOS mRNA ($F_{2,9}$ = 0.879, P = 0.448) and protein ($F_{2,9}$ = 0.081, P = 0.923) in rats treated with DAHP or BH4. **(E,F)** The expression of spinal nNOS mRNA ($F_{2,9}$ = 0.223, P = 0.804) and protein ($F_{2,9}$ = 0.131, P = 0.879) in rats treated with DAHP or BH4. Data are shown as mean ± SEM (n = 4). *Significant difference with respect to vehicle groups (one-way ANOVA followed by Bonferroni *post hoc* test or Dunnett's T3 test if necessary). *$P < 0.05$ and **$P < 0.01$. BH4, tetrahydrobiopterin; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase.

synthesis end product BH4. Besides, BH4 has been implicated to play a crucial role in the distinct peripheral inflammatory pain and neuropathic pain models. Based on these previous findings, we designed to detect the level of plasma and spinal BH4 in rats exposed to chronic RS to validate whether no injury stress-induced hyperalgesia is dependent on the BH4 pathway. Following the procedure of RS, both the plasma BH4 and spinal BH4 concentrations were increased. To further determine whether BH4 could exacerbate pain sensitivity, we intrathecally injected its active enantiomer 6(*R*)-5,6,7,8-BH4 dihydrochloride. Consistent with the previous reports and our assumption, intrathecal administration of BH4 at a dose of 10 μg enhanced mechanical allodynia and thermal hyperalgesia in stressed rats and produced a rapid and long-lasting pain in normal control rats. To sum up, these findings reasonably suggest that the BH4 pathway is associated with the SIH and GCH1 and might be a function gene therapy for chronic pain. Nevertheless, the underlying mechanisms of BH4 modulating the development and maintenance of hyperalgesia are intriguing issues to clarify in further studies.

Studies from the last decades also demonstrated that in either the central mechanisms or the peripheral mechanisms do distinct neurotransmitters (e.g., norepinephrine, 5-hydroxytryptamine, 5-hydroxyindoleacetic acid, dopamine, and NO) play a crucial fundamental role during the nociceptive afferent and descending pain facilitation (Khasar et al., 2009; Kumar et al., 2010; Donello et al., 2011). Several evidence lines have demonstrated that BH4 is an essential cofactor for tyrosine hydroxylase, phenylalanine hydroxylase, tryptophan hydroxylase, and three distinct isoforms of NOS (Nandi et al., 2005; Vyas-Read et al., 2007). Based on the previous studies, we reasonably speculated that BH4 is the common messenger to mediate these neurotransmitters in the SIH pathway. Notably, we observed robust increases of nitrite and nitrate, widely represented as NO production indicators in stressed rats. Furthermore, to validate whether BH4 enhances nociceptive responses partly through the NO

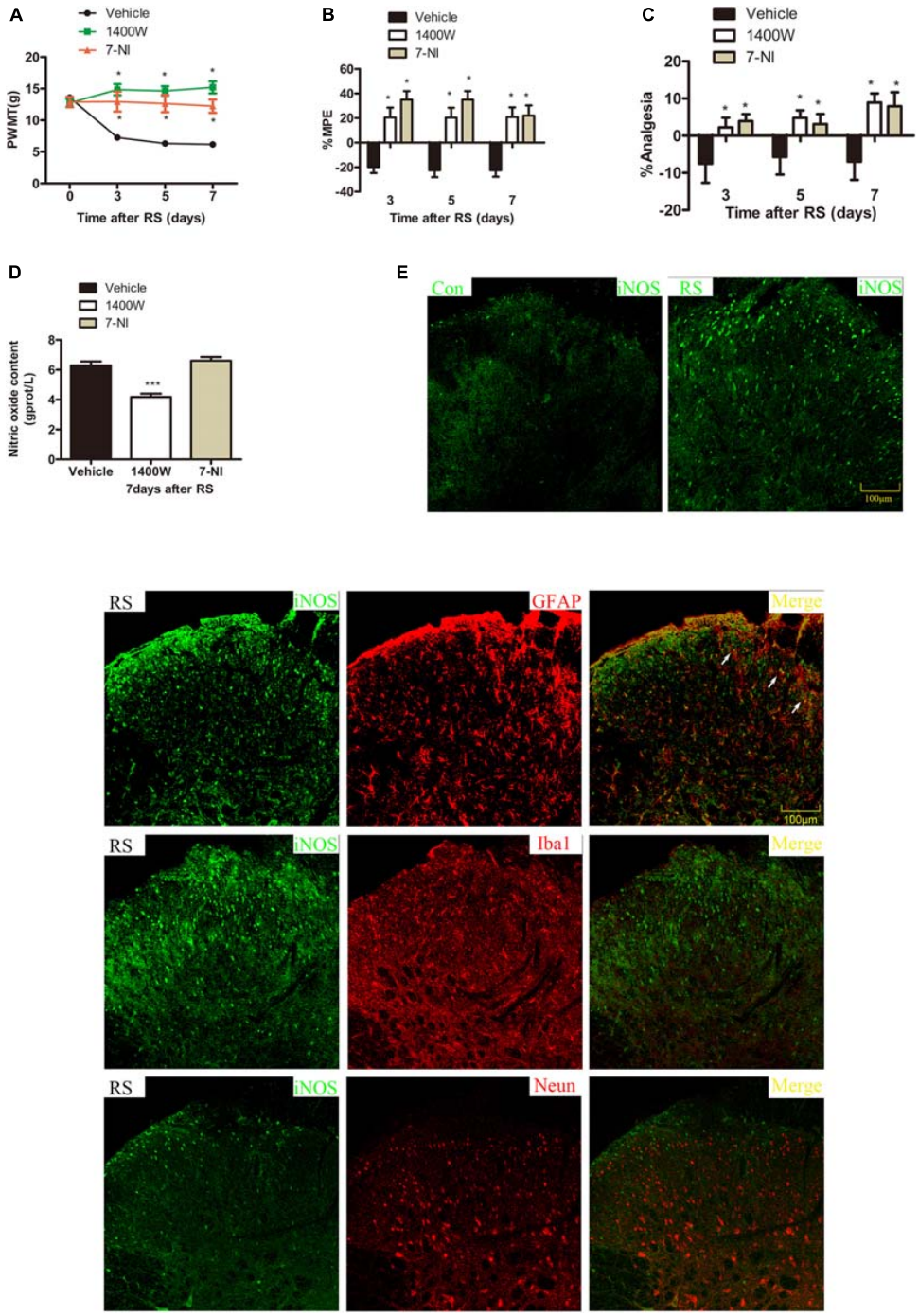

FIGURE 7 | Effect of specific 1400 W or 7-NI on nociceptive behavior and spinal NO in rats subjected to chronic RS, and the cell type specificity of iNOS. Chronic RS rats were consecutively treated with either 1400 W (1 μg/ml, 10 μl) or 7-NI (40 μg/ml, 10 μl). **(A–C)** Nociceptive behavior tests including PWMT (treatment: $F_{2,25} = 20.961$, $P < 0.001$; observation intervals: $F_{3,75} = 4.045$, $P = 0.016$; interaction: $F_{6,75} = 9.247$, $P < 0.0001$), PWTL (treatment: $F_{2,25} = 15.561$, $P < 0.001$; observation intervals: $F_{2,50} = 2.038$, $P = 0.154$; interaction: $F_{4,50} = 1.831$, $P = 0.158$), and TFL (treatment: $F_{2,25} = 4.898$, $P = 0.016$; observation intervals: $F_{2,50} = 3.174$, $P = 0.064$; interaction: $F_{4,50} = 1.1$, $P = 0.362$). **(D)** Effect of 1400 W or 7-NI on the expression of spinal NO, $F_{2,15} = 27.36$, $P < 0.001$. Data are shown as mean ± SEM (n = 8–10). *Significant difference with respect to vehicle groups (two-way ANOVA with repeated measures in nociceptive behavior tests and one-way ANOVA in spinal NO measurement followed by Bonferroni *post hoc* test or Dunnett's T3 test if necessary), $^{*}P < 0.05$ and $^{***}P < 0.001$. **(E)** In the superficial layer of the spinal dorsal horn (lamina I–III), iNOS-positive cells were increased in the RS rats compared with control rats. **(F)** Confocal images of iNOS immunostaining (green) and its colocalization with astrocytes (GFAP, red), but not with microglia (Iba1, red) or neurons (Neun, red) in the superficial spinal dorsal horns (lamina I–III, n = 3 in each group). Scale bar = 100 μm. 7-NI, 7-nitro indazole; NO, nitric oxide; RS, restraint stress; iNOS, inducible nitric oxide synthase; PWMT, paw withdrawal mechanical threshold; PWTL, paw withdrawal thermal latency; iNOS, inducible nitric oxide synthase.

pathway, we detected the expression of spinal NO metabolites in the BH4- and DAHP-treated stressed rats. Consistent with our hypothesis, NO metabolites were significantly decreased after DAHP treatment. Interestingly, only a slight increase of NO metabolites was observed after the administration of BH4 as compared with those in the vehicle rats, which is not consistent with the previous report (Tegeder et al., 2006). The controversial results might be due to the different preclinical models and the time point of observation. L-NAME, a non-selective inhibitor of NO, was widely injected in various pain models, and it effectively attenuated the pain threshold. Our studies demonstrated that pretreatment with L-NAME did attenuate the effect of BH4 on nociceptive responses in stressed rats. Therefore, it is reasonable to clarify that nociceptive effects of BH4 may be exerted via facilitating central sensitization under chronic pain conditions and implicate a vital role of NO pathway in the duration of hyperalgesia in chronic RS (Nasser et al., 2015). Particularly, hyperalgesia effect of NO by facilitating nociceptive transmission might be mediated by the release of glutamate, activation of NMDA glutamate receptor (which increased the c-fos expression and NO synthesis), activation of the guanylyl cyclase-cyclic GMP-PKG pathway, and the phosphorylation of MAP kinase (such as p38 and ERK) at the spinal level (Tegeder et al., 2004; Tang et al., 2007; Quintero et al., 2011). Specifically, it has been reported that NO metabolites were represented as markers of postsynaptic NMDA receptor activation (Quintero et al., 2011). Thus, we speculated that NO-mediated SIH in our studies might respond via the activation of NMDA glutamate receptor, which induced the production and release of NO and in turn modulated presynaptic neurotransmitter release. However, this intriguing issue is required to be answered in the following studies.

Nitric oxide, a soluble gas, as a retrograde messenger modulating the release of various neurotransmitters, is associated with nociception in the peripheral and central nervous systems. NO via the production of constitutive NOS and iNOS could be observed in nervous tissues (Chu et al., 2005; Tang et al., 2007). Previous research suggested that BH4, a cofactor for NOS, could regulate the expression of NOS to produce NO (Tegeder et al., 2006). Remarkably, we showed that SIH induced up-regulation of iNOS, instead of nNOS and eNOS, in SIH mice. DAHP attenuated the pain threshold and down-regulated the expression of iNOS mRNA and protein. Besides, intrathecal treatment of 1400 W (a specific iNOS inhibitor) and 7-NI (a specific nNOS inhibitor) significantly attenuated the hyperalgesia evoked by exposure of RS (Tang et al., 2007), while only a slight decrease in NO metabolites' accumulation is observed in 7-NI-treated SIH mice. These findings corroborate previous reports, indicating that nNOS might compensate for the function of iNOS in SIH (Tao et al., 2003). Nevertheless, our study did not directly clarify the possible role of eNOS in SIH due to the absence of available highly selective inhibitors of eNOS. Thus, we speculated that a substantial amount of NO via iNOS mainly modulated the SIH in chronic RS. These results are consistent with some previous literature (Olivenza et al., 2000; LaBuda et al., 2006; Tang et al., 2007), but contradictory to other reports (Kishimoto et al., 1996; Costa et al., 2005; Tang et al., 2007). The discrepancy might be due to the duration and intensity of stress models. It has been highlighted that iNOS is specifically co-localized with glia, macrophages, and neutrophils in various preclinical models after the stimuli of cytokines, microbial products, or lipopolysaccharide (Moncada et al., 1991; Gross and Wolin, 1995). Under chronic RS conditions, astrocytes in the superficial layer of the spinal dorsal horn become reactive, thus altering morphology and increasing the expression of GFAP, which is widely used as a marker of astrocytes. Moreover, we reported that iNOS was co-expressed with GFAP in the spinal cord instead of Iba1 and NeuN. To the best of our knowledge, there is no study reporting the cell type expressing iNOS in spinal. We speculated that activated astrocytes release a variety of pro-inflammatory cytokines (e.g., tumor necrosis factor-α and interleukin-1β) and neurotransmitters (e.g., NO) (Frank et al., 2007), which, therefore, induces neuronal sensitization, and future detailed study on the role of iNOS induced by BH4 in chronic RS model is required. In summary, the iNOS–NO cascade system partly mediated the SIH at the spinal level. Nevertheless, the underlying mechanisms of iNOS activation responsible for the spinal cord's neurodegenerative changes are needed to clarify in further studies.

Finally, our study indicates the methylation locations of GCH1 in preclinical studies and, thus, may propose a more effective pain therapeutic approach and potential targets for analgesic drugs. Preclinical studies also show that BH4 inhibition with GCH1 by DAHP might occur through competition for the substrate GTP-produced antinociception effects in rats exposed to repeated RS, and BH4 enhances nociceptive responses partly mediated by prevention of excess NO production, suggesting that BH4 has a crucial role in the SIH. Further investigation is essential to understand the precise mechanisms of the BH4 pathway in SIH conditions, as it is reported that chronic RS induces hyperalgesia in male rats instead of female rats (Gamaro et al., 1998). The intriguing issue is required to be answered in further studies. Based on the current findings, our studies may contribute to a better understanding of chronic pain and may provide a more theoretical basis for the therapeutic drug approach in chronic pain.

MATERIALS AND METHODS

Animal Grouping and Treatment

Pathogen-free, male Sprague–Dawley (SD) rats, weighing 190–220 g, and supplied by the Experimental Animal Center of Hubei Province, Tongji Medical College, Huazhong University of Science and Technology (HUST), were housed under a standard temperature (22 ± 2°C) room with standard rodent chow and water available *ad libitum*. All experiments were performed under a protocol approved by the Animal Care and Use Committee of HUST and were conducted following the National Institutes of Health Guide and Ethical Issue of the International Association for the Study of Pain. The animals were subjected to a 12-h light/dark cycle (lights on at 07:00 AM and off at 07:00 PM) maintained under constant conditions for 7 days before the experiment.

The animals were randomly assigned to nine groups: (1) control group (n = 12) with no intervention; (2) RS group (n = 12), in which 6 h (9:00 AM to 03:00 PM) of RS was conducted; (3) vehicle/RS group (n = 8), in which 20 μl of normal saline (NS) was delivered via an intrathecal catheter 15 min before RS; (4) BH4/RS group (n = 10), in which 10 μl (1 μg/μl) of BH4 was given and 10 μl of NS was used for flushing; (5) DAHP/RS group (n = 8), in which 10 μl (6 mg/kg) of DAHP was injected with 10 μl of NS flushing the PE-10 tube; (6) L-NAME/RS group (n = 9), in which 10 μl (30 μg/μl) of L-NAME was injected with 10 μl of NS; (7) L-NAME/BH4/RS group (n = 10), in which 10 μl (30 μg/μl) of L-NAME was injected 30 min before BH4 (1 μg/μl, 10 μl) was injected; (8) 1400 W/RS group (n = 10), in which 10 μl (1 μg/μl) of specific inhibitor of iNOS, 1400 W, was injected into the subarachnoid space with 10 μl of NS flushing the tube; and (9) 7-NI/RS group (n = 10), in which 10 μl (40 μg/μl) of specific inhibitor of nNOS, 7-NI, was injected into the subarachnoid space.

Drugs

Tetrahydrobiopterin, 7-NI, 1400W, L-NAME, DAHP, and dimethyl sulfoxide (DMSO) used in the research were purchased from Sigma–Aldrich Co., United States. BH4 and L-NAME were dissolved in NS and administrated by intrathecal injection. 7-NI, 1400 W, and DAHP were first dissolved in DMSO and then diluted with NS to the desired concentration. All drugs were prepared immediately before administration and given in a volume of 10 μl with 10 μl of NS flushing the PE-10 tube. The dosage was based on Sung et al. (2004), Tegeder et al. (2006), and Makuch et al. (2013).

Intrathecal Catheter Implantation

The method for intrathecal catheter implantation followed the steps in the previous laboratory reports (for details, see **Supplementary Material**) (Ke et al., 2013; Guan et al., 2015).

Restraint Stress Model

Restraint stress was performed according to a previously described chronic RS model. The chronic RS was performed using a plastic tube (18 × 5 cm). Male SD rats (190–230 g) in the RS group were arranged on a plastic tube, leaving enough vents, with an iron clamp from outside penetration clamped tails, in order to adjust the position, avoiding visible physical damage for 6 h (from 09:00 AM to 03:00 PM) (Magarinos and McEwen, 1995). The non-RS animals were maintained in their home cage. Food and water were removed during the time that the RS rats were kept in the plastic tube. Drug administration was 15 min before the RS. Rats were repeatedly exposed to daily RS for seven consecutive days and put back to the home cage with standard rodent chow and water available *ad libitum* (**Figure 8**).

Behavioral Assessments

Nociceptive tests such as the PWMT test, PWTL, and tail immersion test were performed as literature previously (for details, see **Supplementary Material**) (Taliyan and Sharma, 2012; Deciga-Campos et al., 2016).

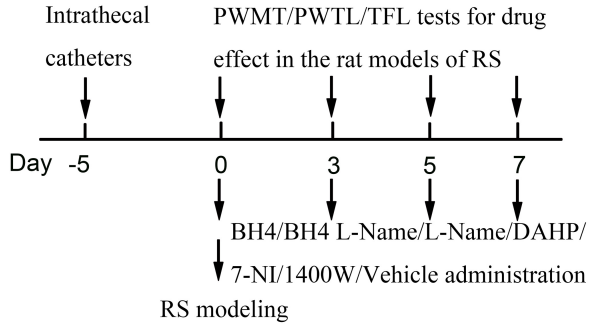

FIGURE 8 | Experimental design. RS, restraint stress; PWMT, paw withdrawal mechanical threshold; PWTL, paw withdrawal thermal latency; TFL, tail-flick latency; BH4, tetrahydrobiopterin; L-NAME, N^{ω}-nitro-L-arginine methyl ester; DAHP, 2,4-diamino-6-hydroxypyrimidine; 7-NI, 7-nitro indazole; 1400W, *N*-([3-(aminomethyl)phenyl]methyl)ethanimidamide.

Western Blot Analysis

Western blot analysis was performed as reported previously (for details, see **Supplementary Material**) (Tegeder et al., 2006; Guan et al., 2015).

Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction

The qRT-PCR analysis was performed as reported previously (for details, see **Supplementary Material**) (Chen et al., 2014).

Immunofluorescence and Immunohistochemistry

Immunofluorescence and immunohistochemistry were performed as reported previously (for details, see **Supplementary Material**) (Guan et al., 2015).

Nitric Oxide Production Assay

Immunofluorescence and immunohistochemistry were performed as reported previously (for details, see **Supplementary Material**) (Prast and Philippu, 2001).

Measurement of the Tetrahydrobiopterin in the Spinal Cord and Plasma

High-performance liquid chromatography analysis was performed as reported previously (for details, see **Supplementary Material**) (Fukushima and Nixon, 1980; Matei et al., 2006; Fekkes and Voskuilen-Kooijman, 2007).

GTP Cyclohydrolase 1 Methylation

GCH methylation analysis was measured as reported previously (for details, see **Supplementary Material**) (Li et al., 2012).

Statistical Analysis

All statistical analyses were conducted using SPSS version 19.0 software (Chicago, IL, United States) and presented as the

mean ± standard error of the mean (SEM). Data from the western blot, RT-PCR assays, etc., were analyzed using Student's *t*-test to compare two groups or one-way analyses of variance (ANOVAs) for multiple comparisons followed by Bonferroni *post hoc* test or Dunnett's T3 tests, if necessary. Data from the nociceptive behavior tests were evaluated using two-way ANOVA with repeated measures, followed by Bonferroni *post hoc* test or Dunnett's T3 test, if necessary. $P < 0.05$ was considered statistically significant.

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 Animal Care and Use Committee of HUST.

AUTHOR CONTRIBUTIONS

YH and HX designed the study and wrote the protocol. BJ, BZ, BX, PL, LA, NY, and YZ performed all the experiments. YH, BJ, and HX undertook the statistical analysis and wrote the first draft of the manuscript. All authors have approved the final manuscript.

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ORIGINAL RESEARCH
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Behaviors Related to Psychiatric Disorders and Pain Perception in C57BL/6J Mice During Different Phases of Estrous Cycle

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Robust sex difference among humans regarding psychiatry- and pain-related behaviors is being researched; however, the use of female mice in preclinical research is relatively rare due to an unchecked potential behavioral variation over the estrous cycle. In the present study, a battery of psychiatry- and pain-related behaviors are examined under physiological condition in female C57BL/6J mice over different estrous cycle phases: proestrus, estrous, metestrous, diestrous. Our behavioral results reveal that there is no significant difference over different phases of the estrous cycle in social interaction test, sucrose preference test, tail suspension test, open field test, marble burying test, novelty-suppressed feeding test, Hargreaves thermal pain test, and Von Frey mechanical pain test. These findings implicate those psychiatry- and pain-related behaviors in normal female C57BL/6J mice appear to be relatively consistent throughout the estrous cycle; the estrous cycle might not be a main contributor to female C57BL/6J mice's variability of behaviors.

Keywords: estrous cycle, female mice, psychiatry, pain, behavior

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INTRODUCTION

Animal models and behavioral tests have been used in experimental research for a long time to increase human knowledge and contribute to finding solutions to the biological and biomedical questions in ways that would be impossible in human beings (Krishnan et al., 2007; Jones et al., 2011; Esquerda-Canals et al., 2017). A dilemma in animal research is that almost all animal models and behavior tests were established and performed in male subjects (Will et al., 2017); however, diseases/symptoms, such as depression and pain, occurred more frequently in women

and manifested differently in men (Kornstein et al., 2000; Calipari et al., 2017; GBD 2016 Disease and Injury Incidence and Prevalence Collaborators, 2017; Kuehner, 2017; Li and Graham, 2017). Lacking mechanistic underpinning of diseases/symptoms in female subjects might be one reason accounting for the unsatisfactory progress of current preclinical research-based translational therapeutics (Wittchen et al., 2002; Steiner et al., 2003; Jang and Elfenbein, 2019). In this case, the National Institutes of Health (NIH) of the United States raised concerns, in 2014, regarding an over-reliance on male subjects in preclinical researches, which aimed to encourage researchers to balance sex in their following cell and animal studies and grant applications (Clayton and Collins, 2014). The naturally occurring estrous cycle has been considered a significant contributor to the variation of behavioral phenotypes in female animals, leading to a serious consideration of estrous cycle effects in future studies. Early studies conducted few decades ago observed the positive impact of the estrous cycle on animal behaviors in different behavioral paradigms (Wang, 1923; Burke and Broadhurst, 1966; Guttman et al., 1975), such as measurements in the revolving wheel test and open field test (Wang, 1923; Burke and Broadhurst, 1966). The estrous cycle is also considered to play a contributing role in affecting the neuronal morphology, network activity, and function modulation in rodents (Maswood et al., 1999; Perez et al., 2014; Lisofsky et al., 2015; Richard et al., 2017), and recently, this phenomenon has re-gained increasing attraction following NIH's announcement. In the field of psychiatry, the first batch of studies using female subjects to establish the mouse model of depression came out sequentially, two of which are chronic social defeat paradigms established on the basis of artificially induced aggression of CD1 mice to the C57BL6J female mice and the other is the vicarious social defeat model (Bourke and Neigh, 2012; Takahashi et al., 2017; Harris et al., 2018; Iñiguez et al., 2018). All of these studies have taken the effect of the estrous cycle on the behavioral outputs observed in consideration (Bourke and Neigh, 2012; Takahashi et al., 2017; Harris et al., 2018; Iñiguez et al., 2018), though none of which reported sufficient effect of estrous cycle to significantly change the performance in social interaction test. On the contrary, social interaction behavior was found to be influenced by estrous cycle in a methylazoxymethanol acetate model of schizophrenia in female rats (Perez et al., 2019). Divergent behavioral outcomes were possibly due to the difference of species, behavioral paradigms, and technical details, which made the effects of estrous cycle on animal behaviors vague and unclear. The contribution of natural estrous cycle stages to animal behaviors is still an open question and needs further investigations for studies in female animals, especially in those widely used ones.

In the present study, based on our research interest, we plan to observe psychiatry- and pain-related behavioral outputs in normal female C57BL/6J mice over different stages of estrous cycle with a battery of widely used experimental paradigms: Social interaction test, sucrose preference test, tail suspension test, open field test, marble burying test, novel environment feeding test, Hargreaves thermal test, and Von Frey mechanical test.

MATERIALS AND METHODS

Materials

Animals

C57BL/6J female mice (7–8 weeks) employed in the present study were provided by Jackson Laboratory (Shanghai) through the Experimental Animal Center of Xuzhou Medical University. Before experimental tests, mice were group-housed and maintained on a 12-h light/dark cycle with food and water available *ad libitum*. All experimental protocols were approved and in accordance with the Animal Care and Use Committee of Xuzhou Medical College (Xuzhou, Jiangsu Province, China). To reduce the number of animals used in the study, some of the mice were reused in different experimental tests. The paw withdrawal latencies (PWLs) and 50% paw withdrawal threshold (50% PWTs) were tested in the same cohort of animals on the same day. The open field test and social interaction test were performed in the same cohort of animals with an interval of 7 days, to allow the animals to recover from the previous experiment's stress. The marble burying test and novelty-suppressed feeding test (NSFT) were performed in the same cohort of animals with an interval of 7 days. To avoid the impact of tests with severe stress on animal behaviors or neuronal activity, two independent cohorts of animals were used to perform tail suspension test or sucrose preference test, respectively.

Identification of the Estrous Cycle

The estrous cycle stage was determined with vaginal smear after behavior test as described previously by McLean et al. (2012). To this end, a micropipette with ∼100 μl sterile autoclaved double distilled water (ddH2O) was used to collect vaginal cells by gently expelling a quarter to half of the volume of water at the opening of the vaginal canal. This procedure was repeated for four–five times to collect a sufficient number of cells. The liquid was then transferred onto a glass slide and the smear was allowed to dry at room temperature completely. The slides were subjected to staining with 0.1% crystal violet (for 1 min followed by two times of wash with ddH_2O). According to the relative ratio of vaginal cell types under light microscopy, the estrous cycle was divided into four phases: proestrus, estrous, metestrous, and diestrous. Briefly, proestrus smear contains only nucleated and cornified cells; metestrous smear contains exclusively cornified cells and an excessive number of leukocytes with relatively few nucleated and cornified cells; and during the diestrous phase, relatively less abundant leukocytes were accompanied by few nucleated and cornified cells. The vaginal cells of each mouse were collected immediately after behavior tests to identify the phase of estrous cycle.

Open Field Test

The open field test (OFT) was performed as we previously reported (Liu et al., 2013; Cho et al., 2017), except for minor modifications in the facility size and test time. Mice were individually placed in a white plastic open-field apparatus (40 cm × 40 cm × 50 cm), which was divided into 3 × 3 subareas as one center area, four corner areas, and four side

areas, illuminated by a 30-W white fluorescent light 2 m overhead. During the test, mice were placed in the center of the open apparatus from the same direction each time. After 2 min of adaptation, the total locomotor activity (time spent in different areas and distance traveled) of each animal was recorded automatically with the ANY-maze tracking system for 3 min.

Sucrose Preference Test

Sucrose preference test (SPT) was performed as we previously reported (Wu et al., 2020). Briefly, mice were individually housed to habituate drinking water with two bottles fitted with ball-point sippers for 2 days. On the testing day, the liquid in one of the bottles with 1% sucrose and both bottles were weighed. Twelve hours later, the position of the bottles was switched to avoid the development of a side preference. At the time point of 24 h, the bottles were weighed for the second time. Sucrose preference was calculated as a percentage of sucrose solution consumption (amount of sucrose solution consumed × 100/total liquid consumption)%.

Tail Suspension Test

Tail suspension test (TST) was performed as the method described by our previous report (Yin et al., 2011). The testing mouse was fixed with a rope at a distance of 2 cm from the tail tip and hung on a shelf with its head about 15 cm from the bottom of the testing setup. The moving activity was video-tracked for 7 min, with the data from the last 5 min used for analysis. Immobility was defined as the cessation of any bodily movements. The immobility duration for each subject within the last 5 min was recorded. Five mice that displayed tail climbing were excluded from the analysis.

Social Interaction Test

A two-stage social interaction test (SI) was performed in a square arena (40 cm × 40 cm) with artificially defined interaction zone (14 cm × 26 cm) and corner zones (10 cm × 10 cm) as we previously reported in both male and female mice (Takahashi et al., 2017; Šabanović et al., 2020). In the first test (target-absent), the experimental mouse was allowed to freely explore the arena with an empty wire mesh sleeve (10 cm × 6 cm) in the interaction zone. In the second stage of the test, the experimental mouse was reintroduced into the arena with an unfamiliar CD1 mouse (male) in the mesh sleeve. As we previously reported, the social interaction ratio (SIR) was calculated as time in interaction zone with target/time in interaction zone without target × 100 (Zhang et al., 2019b). Video-tracking software (ANY-maze, version 4.84, Stoelting Co., Wood Dale, IL, United States) was used to measure the amount of time the experimental mouse spent in the interaction zone and corner zones.

Novelty-Suppressed Feeding Test

As we recently reported (Hodes et al., 2015), before testing, the mice fasted for 24 h with water provided *ad libitum*. The experimental device was a standard hamster cage with the bottom covered with 2-cm corncob. A single food pellet was placed on a 10-cm Petri dish, which coved a circular white filter paper (diameter: 10 cm) in the center of the hamster cage. The experimental mouse was introduced into the corner of the cage, illuminated by a 30-W white fluorescent light 2 m overhead. The latency for mice grasping the food pellet with their forepaws and biting was recorded with an artificially controlled timer. As soon as the mice began to eat or failed to eat the food pellet within 10 min, they would be immediately transferred back to their home cage.

Paw Withdrawal Latency

Paw withdrawal latencies (PWLs) were measured with the IITC Plantar Analgesia Meter (IITC Life Science Inc., Woodland Hills, CA, United States) in a double-blinded manner as described in our previous studies (Liu et al., 2011, 2018; Zhang H. et al., 2017; Zhang S. et al., 2017). Mice were placed in transparent acrylic enclosures (10 × 10 × 20 cm) on a glass plate in a temperature-controlled and noise-free room. The mice were allowed to habituate for 1 h before the behavioral test. A heat-producing radiant light source was used to stimulate the plantar surface of the left hind paw. Time from the "light on" to a typical withdrawal or licking of the tested hind paw was recorded as paw withdrawal latency. The basal PWLs were set to 9–15 s by adjusting the radiant light intensity. To prevent tissue damage, the radiant heat illumination was automatically cut off at 25 s. The PWLs were measured for five times/time points/animal with the last three used for analysis.

Fifty Percent Paw Withdrawal Threshold

The measurement of the mechanical paw withdrawal threshold (PWT) was adapted from and carried out with the up-down paradigm as previously described by Chaplan et al. (1994). Mice were acclimatized for 1 h in transparent acrylic enclosures (10 × 10 × 20 cm) on a wire mesh platform in a temperature-controlled and quiet room. A sequence of calibrated Von Frey filaments (0.02, 0.04, 0.07, 0.16, 0.4, 1.0, 2, and 6 g) was chosen. The measurement was initiated with the 0.16-g hair. Each hair was applied perpendicularly to the plantar surface of the left hind paw, with sufficient force to bend the filament, for about 5 s. Lifting, shaking, or licking of the paw indicated a positive response and prompted the next weaker filament. The absence of a paw withdrawal response prompted the use of the next stronger filament. This paradigm continued until a total of six measurements or until four consecutive positive or four consecutive negative responses occurred. The 50% mechanical withdrawal thresholds were calculated as 50% PWT = Power[10,(Xf+κδ)] in a Microsoft Excel (2010) document; Xf = value (in log units) of the final Von Frey hair used, κ = tabular value [see Appendix from reference (Chaplan et al., 1994)] for the pattern of positive/negative responses, and δ = mean difference (in log units) between stimuli (here, 0.411).

Marble Burying Behavior Test

According to the method described previously (Angoa-Pérez et al., 2013), the marble burying test was performed in a standard mouse cage with 20 marbles placed on the 5-cm depth of corncob bedding in 4 × 5 grids. The mouse was allowed 30 min to freely explore and bury marbles with two 30-W white fluorescent lights

3 m overhead. Marbles were considered buried if 2/3 or more of the marble volume was submerged. The numbers of marbles buried was recorded.

Data Analysis and Statistics

GraphPad Prism 7.0 was used for data analysis and figure generation. All data were expressed as the mean ± SEM. Behavioral results with homoscedastic datasets were compared by one-way ANOVA followed by Tukey's test. Data that did not pass the homoscedastic test was analyzed by a nonparametric test (Kruskal–Wallis test). Sample sizes are indicated in the figure legends, and $P < 0.05$ was considered statistically significant.

RESULTS

Identification of the Estrous Cycle in Female C57BL/6J Mice

Cytological components of vaginal smear determined the estrous cycle in female C57Bl/6J mice. In brief, proestrus smear contains only nucleated and cornified cells; metestrous smear contains exclusively cornified cells and an excessive number of leukocytes with relatively few nucleated and cornified cells; and during the diestrous phase, moderately less abundant leukocytes were accompanied by few nucleated and cornified cells (**Figure 1A**). A consecutive examination of the estrous cycle from 19 mice indicated that a typical estrous cycle lasts 4–8 days (**Figures 1B,C**).

Open Field Test

The open field test developed by Calvin Hall is an experimental test extensively used to assay the level of anxiety-like behaviors and general locomotor activity (**Figure 2A**). In our study, the time mice spent in the center [$F_{(3,54)} = 0.71$, $P = 0.54$, one-way ANOVA], side areas [$F_{(3,54)} = 0.70$, $P = 0.55$, one-way ANOVA], and corner subareas [$F_{(3,54)} = 0.11$, $P = 0.95$, one-way ANOVA] over four estrous stages did not exhibit significant difference (**Figures 2B–E**). In order to evaluate the effect of the estrous cycle on animal's locomotor activity, the total distance that mice traveled during the test was analyzed [$F_{(3,54)} = 0.09$, $P = 0.96$, one-way ANOVA], and no difference was observed between any two different stages (**Figure 2F**).

Social Interaction Test

The two-stage social interaction test is an effective and widely used measurement to indicate pathology-related susceptibility to defined stress (**Figure 3A**), such as social avoidance in rodent models of major depressive disorder and autism disorder. In brief, decreased time in the interaction zone or increased time in corner zones indicated pathology-related social avoidance or susceptibility to defined stress. Under the present condition, we failed to observe difference in interaction zone time [target absent, $F_{(3,48)} = 0.7291$, $P = 0.5397$; target present, $F_{(3,48)} = 0.1686$, $P = 0.9171$, one-way ANOVA], corner zone time [target-absent, $F_{(3,48)} = 0.75$, $P = 0.5277$; target present, $F_{(3,48)} = 0.6966$, $P = 0.5587$, one-way ANOVA], and SIR [$F_{(3,48)} = 0.0897$, $P = 0.9654$, one-way ANOVA] over four estrous stages (**Figures 3B–G**). Additionally, the distance traveled [target absent, $F_{(3,48)} = 1.29$, $P = 0.2885$; target present, $F_{(3,48)} = 1.11$, $P = 0.3542$, one-way ANOVA] and mean movement velocity [target absent, $F_{(3,48)} = 2.027$, $P = 0.1226$, one-way ANOVA; target present, $H = 3.18$, $P = 0.3647$, Kruskal–Wallis test] of female mice also did not vary across phases of the estrous cycle (**Figures 3H,I**). Moreover, six animals were removed from the dataset because the significant outliers (SIR ≥ 600).

Marble Burying Behavior Test

Marble burying is an animal behavioral test used in scientific research to depict anxiety-like behaviors, repetitive, or obsessive-compulsive disorder behavior. It is based on the observation that rats and mice will bury either harmful or harmless objects in their bedding. In the present study, our observation revealed that the number of buried marbles did not differ significantly at different phases of the cycle ($F_{(3,\ 46)} = 0.2724$, $P = 0.8450$, one-way ANOVA, **Figure 4**). The above result indicated that there is no detectable correlation between the estrous cycle and repetitive and compulsive-like behaviors in the marble burying test.

Novelty-Suppressed Feeding Test

Novelty-suppressed feeding test assesses the ability of the animal to resolve a conflict between a context that induces heightened anxiety-like emotionality and a drive to approach an appetitive stimulus. It is also used for assessing the efficacy of potential anxiolytic drugs. In the present study, the latency to eat the food pellet was recorded for mice under different estrous cycle stages. The anxious mice would spend a longer latency to eat. The result indicated that female mice at different stages displayed similar latency to eat [$F_{(3,53)} = 0.05$, $P = 0.98$; one-way ANOVA, **Figure 5**].

Tail Suspension Test

The tail suspension test is a mouse behavioral test useful in screening potential antidepressant drugs and assessing other manipulations that are expected to affect depression-related despair behaviors. Increased immobility time in the tail suspension test suggested a higher level of despair. In our experiment, no difference in immobility time among groups was observed [$F_{(3,47)} = 0.5166$, $P = 0.6728$, one-way ANOVA, **Figure 6**]. The result demonstrated that the estrous cycle did not affect depression-like behavior in the tail suspension test.

Sucrose Preference

The two-bottle choice procedure for assessing sucrose preference is a useful test to investigate anhedonia (inability to feel pleasure, a core symptom of depression in humans) in laboratory rodents, particularly in stress-based models of depression. Across the four different estrous stages, the female experimental mice exhibited a similar preference to 1% sucrose solution [$F_{(3,65)} = 1.16$, $P = 0.33$, one-way ANOVA, **Figure 7**], which supported that the estrous cycle was not a contributor to sucrose solution-related anhedonia.

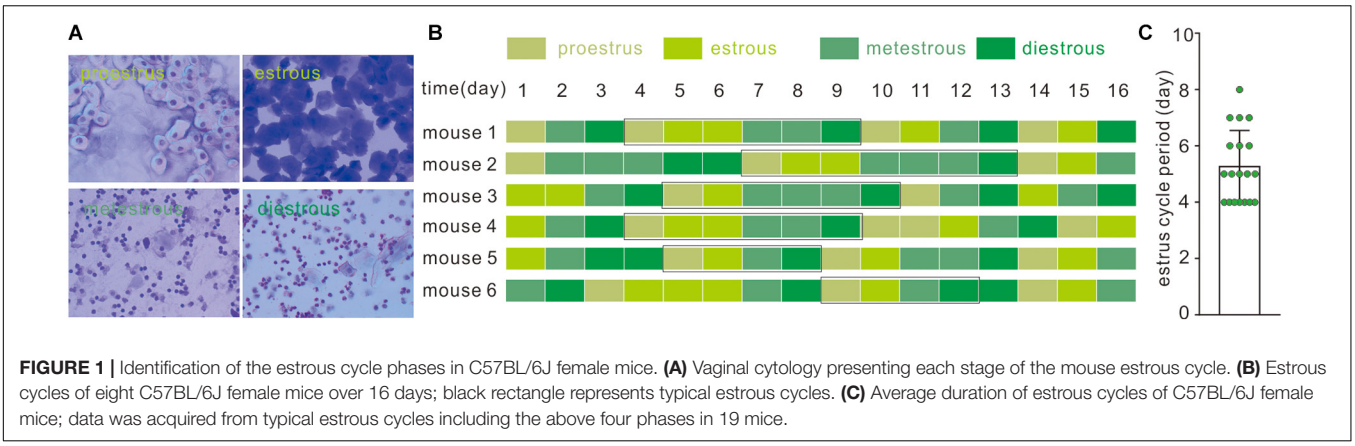

FIGURE 1 | Identification of the estrous cycle phases in C57BL/6J female mice. **(A)** Vaginal cytology presenting each stage of the mouse estrous cycle. **(B)** Estrous cycles of eight C57BL/6J female mice over 16 days; black rectangle represents typical estrous cycles. **(C)** Average duration of estrous cycles of C57BL/6J female mice; data was acquired from typical estrous cycles including the above four phases in 19 mice.

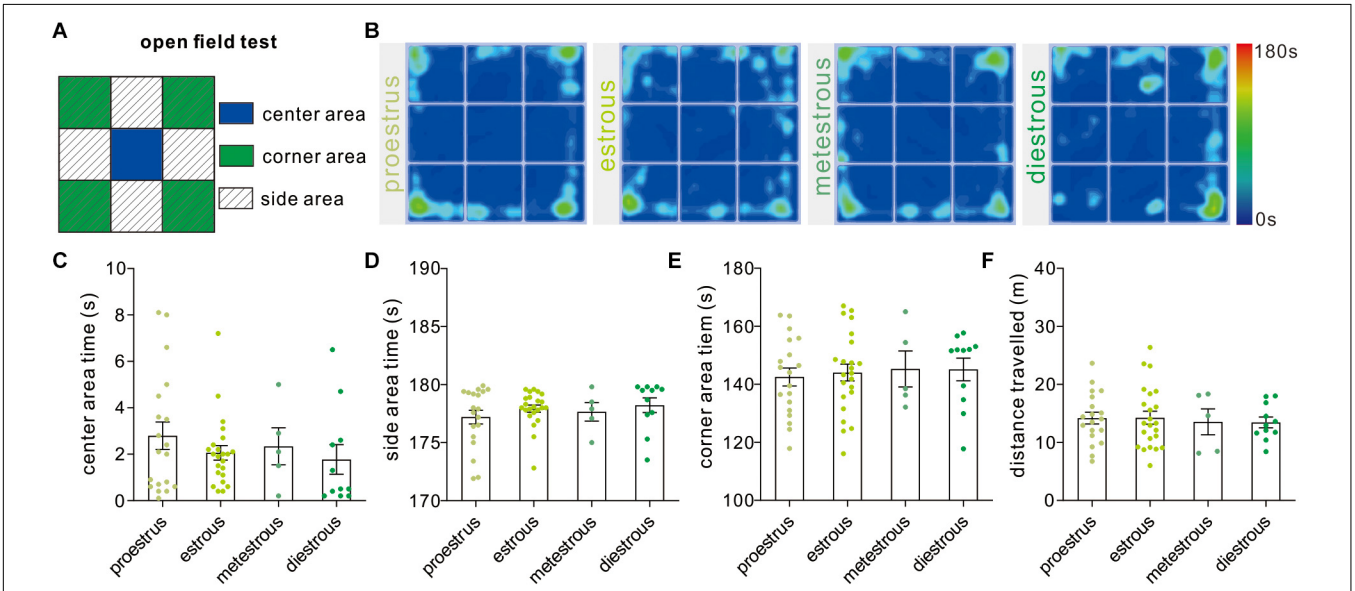

FIGURE 2 | Open field test. **(A)** The schematic diagram showing center, corner, and side areas of the open field. **(B)** Representative heat maps showing time spent in each area of the open field chamber. **(C–E)** Open field analysis showing no significant difference in time spent in the center, side, and corner areas at different phases (n = 19, 23, 5, and 11). **(F)** Total distance traveled over the whole apparatus under different estrous cycle phases (n = 19, 23, 5, and 11).

Pain-Related Behaviors

Hargreaves test and Von Frey test were used to measure thermal nociceptive sensitivity and mechanical nociceptive sensitivity by quantitatively evaluating the paw withdrawal latency to noxious thermal stimulation and 50% paw withdrawal threshold to a non-noxious mechanical stimulation. In both tests, our behavioral results revealed no difference in PWLs [$F_{(3,57)}$ = 1.32, P = 0.27, one-way ANOVA, **Figures 8A,B**] and 50% PWTs (H = 3.38, P = 0.3367, Kruskal–Wallis test, **Figures 8C,D**) in female mice among groups.

DISCUSSION

In this study, utilizing a battery of widely accepted behavioral tests, we examined the estrous cycle effects on a number of psychiatry- and pain-related animal behaviors in female C57BL/6J mice. Our results suggest that the behaviors described above stayed unchanged throughout different stages of the estrous cycle.

Clinical experience and scientific researches consistently agree that male and female individuals do generally differ at baseline and in response to the exposure of external stress and noxious stimuli. However, the majority of preclinical and clinical studies have been carried out in male individuals, initially due to the perception that data acquired in females under different stages of the estrous cycle would be more varied than that obtained from males. Estrogen, a hormone that fluctuates over estrous cycle, and the agonist of its receptors has also been implicated in modulating animal behaviors in a large number of studies (Crider et al., 2018; Popov et al., 2020; Qu et al., 2020; Renczés et al., 2020; Ghazvini et al., 2021; Valdés-Sustaita et al., 2021). Consistently, preclinical studies discovered a statistical difference in psychiatry- and pain-related behavioral and physiological traits (Meziane et al., 2007).

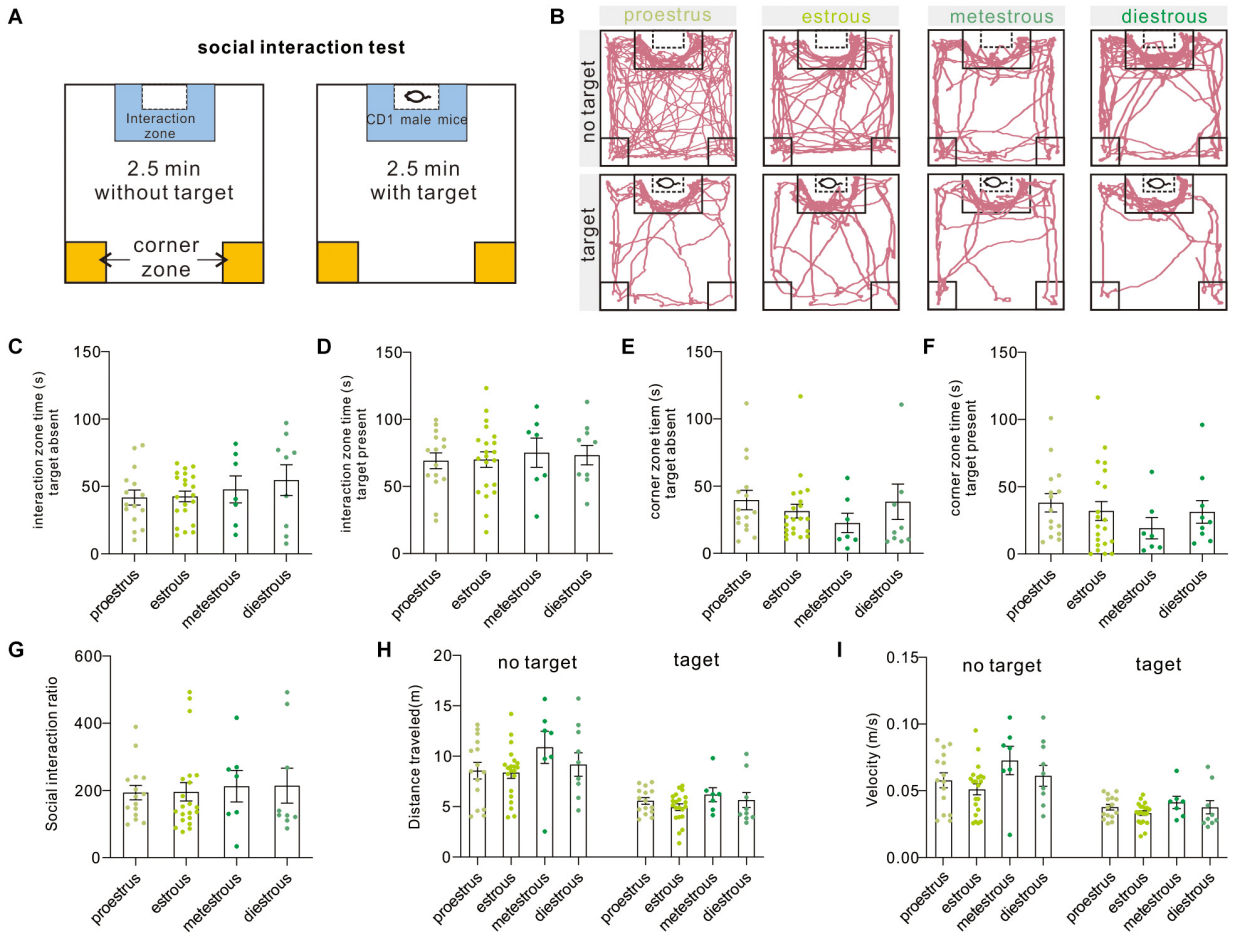

FIGURE 3 | Social interaction test. **(A)** Schematic overview of the social interaction arena and behavioral test procedure. **(B)** Representative moving tracks of test mice during social interaction test at different phases of the estrous cycle. **(C,D)** Interaction zone time in the presence or absence of an unfamiliar CD1 mouse (n = 15, 21, 7, and 9). **(E,F)** Corner zone time in the presence or absence of an unfamiliar CD1 mouse (n = 15, 21, 7, and 9). **(G)** SIR of female mice at different estrous cycles (n = 15, 21, 7, and 9). **(H)** Distance traveled in the presence or absence of an unfamiliar CD1 mouse (n = 15, 21, 7, and 9). **(I)** Mean velocity in the presence or absence of an unfamiliar CD1 mouse (n = 15, 21, 7, and 9). SIR, social interaction ratio.

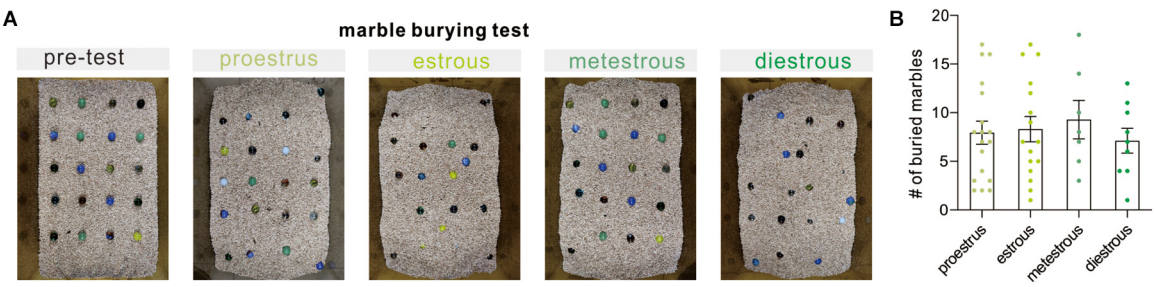

FIGURE 4 | Marble burying behavior test. **(A)** Representative examples of the marble burying test arena following the test. **(B)** Quantitative data of buried marble numbers at different estrous cycles over a 30-min period (n = 18, 16, 7, and 9).

For example, few strains of female mice were reported to display varied behaviors over different cycling stages in the forced swim test, social interaction test, tail suspension test, and pain-related behavioral tests (Meziane et al., 2007; Rebolledo-Solleiro and Fernández-Guasti, 2018; Chari et al., 2020). Similar behavioral fluctuations were also observed in rats in early studies (Wang, 1923; Burke and Broadhurst, 1966; Guttman et al., 1975). The effect of the estrous cycle on neuronal and molecular physiology in a few brain areas was also supported by electrophysiological studies (Calipari et al., 2017; Jaric et al., 2019). Surprisingly, few

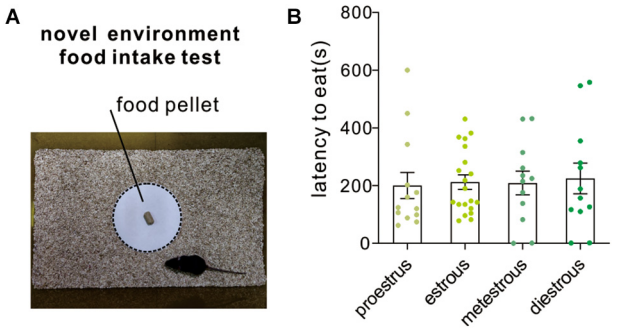

FIGURE 5 | Novelty-suppressed feeding test. **(A)** An image shows the testing arena and food platform used for novel environment food intake test. **(B)** Quantitative data of feeding latency at different estrous cycles (n = 13, 20, 12, and 12).

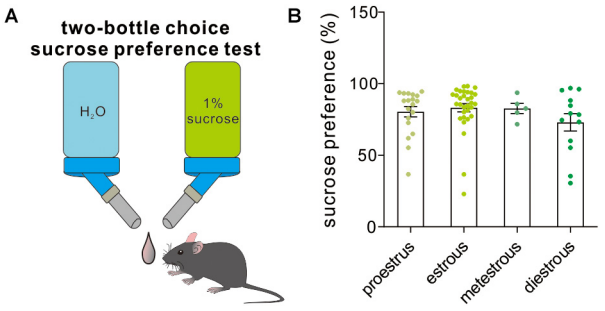

FIGURE 7 | Two-bottle choice sucrose preference test. **(A)** A schematic cartoon showing two-bottle choice sucrose preference test. **(B)** Quantitative data of sucrose preference during a 12-h testing period under different estrous cycles (n = 19, 32, 5, and 13).

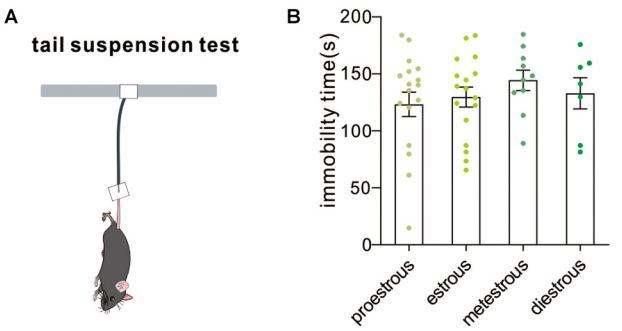

FIGURE 6 | Tail suspension test. **(A)** A schematic cartoon showing tail suspension test setup. **(B)** Quantitative data of not-moving time during a 5-min tail suspension test at different estrous cycles (n = 17, 17, 10, and 7).

recent meta-analysis of preclinical data surprisingly indicated that the variance in data obtained from female rodents under distinct cycling stages is not different from that obtained from the males, indicating that estrous cycle is possibly not a main contributor to observed behavioral variance (Prendergast et al., 2014; Becker et al., 2016). This evidence leads to a divergence between the unchanged behavioral observations and the unstable physiology over the estrous cycle in female animals. In our view, an interpretation of this divergence is that estrous cycle-related neurobiological changes in specific brain regions is an active adaptation of the nervous system to achieve intrinsic homeostasis and maintain the stability of animal behaviors, which is essential for the survival of species (Zhang et al., 2019a).

Behavioral response to external stimuli under pathological states is more complicated than that under the physiological state. For example, following a repeated social stress model of depression, a subpopulation of mice displayed social avoidance to a novel social target in a social interaction test (susceptibility). Simultaneously, the rest remained in a normal-like social interaction behavioral phenotype (resilience) (Cao et al., 2010; Friedman et al., 2014, 2016; Walsh et al., 2014; Bagot et al., 2015; Zhang et al., 2019b). Similarly, following exposure to repeated tail shocks in a restrainer, half of the rats developed learned helplessness to later shock in a shuttle-box test, whereas the rest of the subpopulation exhibited a similar behavioral phenotype with stress-naive controls, which could also be seen as susceptibility and resilience to stress (Sartorius et al., 2003; Taneja et al., 2011). The behavioral phenotypes following pathological models will be more complicated when mixed with the effect of estrous cycle.

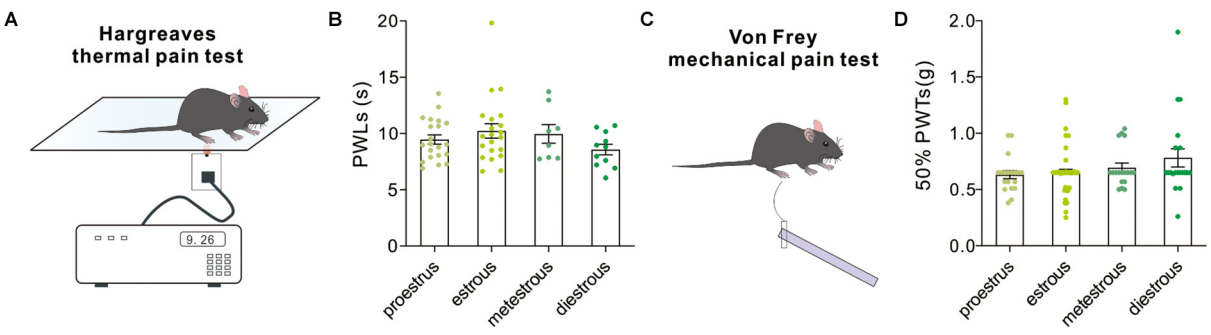

FIGURE 8 | Pain-related behavioral tests. **(A)** A schematic cartoon showing the Hargreaves thermal pain test for paw withdrawal latency to a thermal stimulation. **(B)** Quantitative data of paw withdrawal latencies under different estrous cycles (n = 21, 21, 8, and 11). **(C)** A schematic cartoon showing the Von Frey test for paw withdrawal threshold to mechanical stimuli. **(D)** Quantitative data of 50% paw withdrawal threshold under different estrous cycles (n = 20, 64, 18, and 20).

Moreover, the underlying mechanisms, for example, the active homeostasis in the midbrain dopamine neurons, are even more complicated than the behavioral outcomes. In this case, it would be necessary to look at the effect of the estrous cycle on behaviors following specific animal models.

Our data obtained from the behavioral tests contrasts with the findings observed in a previous research on the female rodents. This study found a significant effect of the Whitten effect-induced estrous cycle on pain behaviors with tail-flick test and hot plate test to thermal stimulation, anxiety-related behaviors in open field test, and despair behavior in tail suspension test, in BALB/cByJ strains, but the estrous cycle of female C57BL/6J mice only affects despair behavior in the tail suspension test (Meziane et al., 2007). In contrast with the data presented by Meziane et al. (2007), utilizing Hargreaves thermal pain test, Von Frey Mechanical pain test, open field, and tail suspension setups, we did not observe any difference of behavior regarding pain over the different phases of the whole estrous cycle. This divergence of the tail suspension test possibly comes from a methodological variability between studies. Taken together, their study and ours indicated that there is a significant difference in the effect of estrous cycle on animal behaviors among mouse strains, and this effect appeared not remarkable in C57BL/6J mice no matter if the estrous cycle is artificially induced or naturally happened.

There are limitations in this study. First, to reduce the number of animals used in this study, some animals were used for two different behavioral paradigms, for example, in the Hargreaves thermal pain test and Von Frey mechanical pain test, in the open field test and social interaction test, as well as in the marble burying test and novelty suppressed feeding test. It is widely accepted that these behavioral tests generate weak, if not, no stress on the test animals, especially after a long-term recovery. However, we could not completely exclude the possibility that the first behavioral test and the virginal smear test that followed would affect the second behavioral test's data. Second, the present study was conducted with a relatively small sample size, though acceptable for studies with male animals, affecting the data collection and interpretation. Third, behavioral data from the first 2 min in the open field test was not included in the data analysis, which might represent as an essential reflection of anxiety-like behaviors.

In summary, our study has suggested that the estrous cycle is not a key effector of variance in psychiatry- and pain-related behaviors in female C57BL/6J mice, and behavioral tests blind to the estrous cycle might be acceptable in naive mice. It is also proposed that the potential interaction between estrous cycle and external stimuli may lead to complex behavioral outcomes; in line with this phenomenon, it is suggested that the future investigation should be more attentive toward the influence of estrous cycle on animal behaviors in pathological states, such as specific animal models that reflect pathological conditions of human diseases.

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 Animal Care and Use Committee of Xuzhou Medical University.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Comorbid Chronic Pain and Depression: Shared Risk Factors and Differential Antidepressant Effectiveness

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The bidirectional relationship between depression and chronic pain is well-recognized, but their clinical management remains challenging. Here we characterize the shared risk factors and outcomes for their comorbidity in the Australian Genetics of Depression cohort study ($N = 13,839$). Participants completed online questionnaires about chronic pain, psychiatric symptoms, comorbidities, treatment response and general health. Logistic regression models were used to examine the relationship between chronic pain and clinical and demographic factors. Cumulative linked logistic regressions assessed the effect of chronic pain on treatment response for 10 different antidepressants**.** Chronic pain was associated with an increased risk of depression (OR = 1.86 [1.37–2.54]), recent suicide attempt (OR = 1.88 [1.14–3.09]), higher use of tobacco (OR = 1.05 [1.02–1.09]) and misuse of painkillers (e.g., opioids; OR = 1.31 [1.06–1.62]). Participants with comorbid chronic pain and depression reported fewer functional benefits from antidepressant use and lower benefits from sertraline (OR = 0.75 [0.68–0.83]), escitalopram (OR = 0.75 [0.67–0.85]) and venlafaxine (OR = 0.78 [0.68–0.88]) when compared to participants without chronic pain. Furthermore, participants taking sertraline (OR = 0.45 [0.30–0.67]), escitalopram (OR = 0.45 [0.27–0.74]) and citalopram (OR = 0.32 [0.15–0.67]) specifically for chronic pain (among other indications) reported lower benefits compared to other participants taking these same medications but not for chronic pain. These findings reveal novel insights into the complex relationship between chronic pain and depression. Treatment response analyses indicate differential effectiveness between particular antidepressants and poorer functional outcomes for these comorbid conditions. Further examination is warranted in targeted interventional clinical trials, which also include neuroimaging genetics and pharmacogenomics

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protocols. This work will advance the delineation of disease risk indicators and novel aetiological pathways for therapeutic intervention in comorbid pain and depression as well as other psychiatric comorbidities.

Keywords: depression, chronic pain, suicide, treatment response, comorbidity, antidepressant

INTRODUCTION

Depression is estimated to affect over 264 million people worldwide and is a leading cause of global disability (1). Its clinical manifestations and outcomes are highly heterogeneous, with multiple factors underlying susceptibility, progression and treatment response (2). One key factor that frequently complicates the diagnosis of depression is comorbid chronic pain, as patients presenting with pain are more likely to be investigated medically rather than as part of a broader biopsychosocial framework (3). Depression and chronic pain frequently coexist, with up to 60% of chronic pain patients also presenting with depression (4, 5). Furthermore, the combination of chronic pain and depression leads to poorer treatment outcomes and overall functioning than either condition alone (6). This problem is underlined by depression and chronic pain being among the top three leading causes of global disability over the past three decades (1).

Chronic pain has been defined by the International Association for the Study of Pain (IASP) as pain persisting or recurring for longer than 3 months (7, 8). In contrast to acute pain, which alerts individuals to potential or real tissue damage, chronic pain serves no apparent physiological purpose and persists beyond normal healing time (7). In Australia (2015–2016), the disease group with the highest expenditure was musculoskeletal disorders (9). In 2016, more than 1.5 million people over the age of 45 had chronic pain (10) and nearly half of adult patients referred to a pain specialist have comorbid anxiety or depression (11). In 2018 the cost to the Australian economy was around $139 billion, mostly due to lost productivity and impaired quality of life (10) with predictions it will almost triple by 2050 (12). In the United States, chronic pain is already costing well over $500 billion per year (13, 14).

The relationship between chronic pain and depression is bidirectional, as having either disorder increases the risk of developing the other condition (15–19) and pain, in particular, is strongly associated with depression onset and relapse (20–26). Furthermore, the relationship is dose-dependent with more severe pain being associated with greater severity of depression (23, 27–31). That is especially true for older age populations which report the highest prevalence (13%) (15) of comorbid chronic pain and depression out of all age groups (3, 32–34). However, the evidence for comorbid psychiatric disorders predicting pain intensity and worse outcomes is much weaker (18, 35). Nevertheless, recent large-scale human genetic studies (36–52), animal models (53–56) and neuroimaging in antidepressant treatment trials (57–59) have made essential inroads toward delineating the causal mechanisms between chronic pain and depression.

Serotonin noradrenaline reuptake inhibitors (SNRIs; e.g., duloxetine) and selective serotonin reuptake inhibitors (SSRIs; e.g., paroxetine, sertraline) are commonly used antidepressants for the treatment of comorbid chronic pain and depression (60). Other antidepressant options include tricyclic antidepressants (TCAs) such as amitriptyline (61, 62). While these medications have been found to reduce the symptoms of both depression and pain partially, no significant differences in efficacy between them have been established so far (63), thus further research is required (60, 64). For example, the efficacy of TCAs against other antidepressants for the treatment of comorbid chronic pain and depression remains unclear due to a lack of rigorous studies (35, 65, 66).

Despite the high prevalence and cost of comorbid chronic pain and depression (1, 4, 5, 15–17, 19, 67–69), research efforts have yet to deliver clinically useful findings and recommendations specifically for this comorbid indication (66, 70, 71). For example, a recent review highlighted that it was unclear which specific antidepressant should be prescribed as the first-line treatment for comorbid chronic pain and depression (60), while others have recommended non-opioid medications as first-line therapy for chronic neuropathic pain (72, 73). To address this issue, pharmacoepidemiological studies—which examine the use and effect of medications in large population cohorts—have been proposed as a cost-effective method for reviewing pharmaceutical safety and effectiveness, as well as helping to inform clinical guideline development (74).

In the current study, we examined the pharmacoepidemiology of comorbid chronic pain and depression in the Australian Genetics of Depression Study (AGDS)—one of the world's largest participant cohorts with a detailed history of depression and its comorbidities (75). Here, we sought to: (i) quantify the association between depression and chronic pain; (ii) assess the dependency between chronic pain severity, depression severity and recent suicidality; (iii) identify other psychiatric disorders and patterns of recent substance use associated with comorbid chronic pain and depression; and (iv) assess whether comorbid chronic pain and depression is associated with differential antidepressant effectiveness.

METHODS

Participants

This study comprised data from two cohorts: AGDS and the Prospective Imaging Study of Aging (PISA). Participants in both groups provided informed consent before participating. These studies, including all questionnaires used, were approved by QIMR Berghofer Medical Research Institute's Human Research Ethics Committee.

AGDS Cohort

Twenty thousand six hundred eighty-nine participants from across Australia were recruited through an open media campaign and targeted mailout. The publicity campaign, from which 86% of participants were recruited, including both conventional and online social media. The campaign appealed for anyone who "*had been treated by a doctor, psychiatrist or psychologist for depression*" to visit this website—https://www.geneticsofdepression.org.au. For the targeted mailout, invitation letters were sent by the Australian Government Department of Human Services (DHS) to individuals who, according to their records, had received at least four prescriptions for any of the 10 most commonly used antidepressants in the last 4.5 years. DHS did not, at any time, share any personal information with the research team. Potential participants were directed to the above website, which contained information about the study, a registration and consent form, and a comprehensive online questionnaire. The essential inclusion criteria included having been prescribed and taken antidepressants and providing consent to donate a saliva sample for subsequent genotyping. No participant was excluded based on comorbid conditions. The online survey assessed mental health diagnoses, antidepressant response, suicidality, general health and substance use, among several other variables. A detailed baseline description of the cohort has been published elsewhere (75). The full list and details of instruments used for AGDS phenotyping are available at: https://bmjopen.bmj.com/content/bmjopen/10/5/e032580/DC2/embed/inline-supplementary-material-2.pdf.

PISA Cohort

The Prospective Imaging Study of Aging (PISA) is a longitudinal cohort of Australian adults (76). The population-based sample recruitment pool comprised adult twins, their spouses, and first-degree relatives of twins and spouses who over previous decades, had volunteered for studies on risk factors or biomarkers for physical or psychiatric conditions and had previously been genome-wide genotyped (77, 78). The PISA protocol consisted of online questionnaires, including a history of mental health diagnoses and the same pain questionnaire in AGDS. It was completed by $N = 2{,}469$ PISA participants. For that reason, AGDS and PISA data were used in the present study to assess the effect of depression and demographics (e.g., age, sex) on chronic pain. All other analyses described in this manuscript were performed only in the AGDS cohort.

Depression and Chronic Pain Ascertainment

AGDS participants were asked to self-report whether they had ever been diagnosed with depression by a health professional, and similarly for 19 other psychiatric conditions. Individuals were classified as depression cases if they had reported both a depression diagnosis and had been prescribed antidepressants in the past 5 years ($N = 17{,}849$). Of these, 92% fulfilled the DSM-5 diagnostic criteria for a lifetime depressive episode based on detailed descriptions of this cohort (75). Importantly, this figure is within the test-retest reliability estimates of depression ascertainment from DSM-5 based instruments (79, 80). Participants were administered a pain severity numerical rating scale (81). Briefly, patients were asked to indicate whether they experienced chronic pain in their daily life and to rank its intensity on a scale from 0 to 10. Only those reporting a pain rating >0 progressed to the remainder of the pain module, which included questions about the duration and location of their primary pain. Following the IASP guidelines, chronic pain was defined as pain persisting or recurring for at least 3 months (7, 8). Cases were classified as having comorbid chronic pain and depression if they fulfilled the criteria for both conditions ($N = 6{,}895$), and *controls* were classified as those who reported depression but no chronic pain ($N = 4{,}475$). We performed a complete case analysis. Thus, participants with missing data for chronic pain (i.e., those who did not complete the section; $N = 6{,}463$) were excluded from analyses that needed data for both chronic pain and depression.

Recent Suicidality and Substance Use

Suicidality was assessed using the SIDAS instrument (82). Briefly, suicidal ideation over the last month was measured on a 10-point scale: 0 indicated having no suicidal ideation in the past month (never), and 10 denoted persistent suicidal ideation. Participants with a score >0 were classified as positive cases for suicidal ideation. Suicide attempt was measured using a similar 10-point scale in regard to how close a participant had come to making an attempt. Only those with a score of 10 (labeled as "*I have made an attempt*") were considered cases for a suicide attempt. Participants also reported their frequency in using a range of substances over the last 3 months. Alcohol consumption frequency was measured as the number of days the participant drank three or more standard drinks. For all other substances, the response options were: "*never*" (0), "*once or twice*" (1), "*monthly*" (2), "*weekly*" (3), or "*daily*" (4). These responses were modeled as continuous variables when assessing their correlation with chronic pain.

Antidepressant Use and Response

Participants were asked whether they had ever been prescribed any of the 10 most commonly used antidepressants in Australia for any indication. These are sertraline, escitalopram, venlafaxine, amitriptyline, mirtazapine, desvenlafaxine, citalopram, fluoxetine, duloxetine and paroxetine. Information regarding the reason(s) for prescription was collected using a checklist of 17 possible responses, including depression, chronic pain, and anxiety (among others). Multiple selections were possible. Participants were asked to report on the best aspects of taking antidepressants using the following item: "*What were the best aspects of taking the antidepressant(s)? Include any antidepressant you have taken.*" Participants were then able to select all that apply out of a list including relief of depressive symptoms, relief of other symptoms, e.g., sleep disturbance, reduction in suicidal ideation, return of normal emotion, improved relationships, returning to normal activities and restored control over mood. Moreover, participants rated the effectiveness of each antidepressant they had taken, using a scale ranging from 0 (e.g., "*sertraline works not at all well for me*") to 2 (e.g., "*sertraline works very well for me*"). Two

analyses were performed: (i) first, antidepressant effectiveness was compared between participants who reported taking an antidepressant prescribed for chronic pain against the rest of the participants (i.e., not prescribed for chronic pain); and (ii) we compared antidepressant effectiveness between participants reporting chronic pain and those reporting no chronic pain (regardless of explicit indication).

Statistical Analyses

In this study, we used complete case analysis and thus removed participants who did not have the required data from specific analyses. The relationship between chronic pain and several other variables of interest was assessed using multivariable logistic regression. This approach enabled us to quantify the associations while adjusting for age, sex and all other relevant factors (e.g., the correlation between alcohol and chronic pain while keeping usage of all other substances *equal*). Fully adjusted odds ratios were calculated from effect sizes on the logit scale, and *p*-values were estimated using Wald-tests. For all analyses, the presence of chronic pain was modeled as a binary variable, while chronic pain severity was modeled as a quantitative score from zero to 10. The relationship between chronic pain and antidepressant effectiveness was examined using *cumulative link logistic regressions* to accurately model treatment response, which was coded on an ordinal scale. Furthermore, to assess the effect of chronic pain across all antidepressants, a random effect was included to account for repeated responses from participants. This analysis was performed in R using the ordinal package and the *clm* and *clmm* functions, adjusting for the effects of sex and age when antidepressant treatment started. All other statistical analyses were performed and figures generated in *python* using these modules: *statsmodels*, *scipy*, *numpy*, *pandas*, *matplotlib,* and *seaborn*.

RESULTS

Sample Demographics and Association Between Chronic Pain and Depression

Demographics and chronic pain prevalence for both AGDS (enriched for depression) and PISA (not enriched for depression) cohorts are shown in **Table 1**, **Supplementary Figures 1, 2**. **Figure 1** shows the prevalence of chronic pain by age, stratified by cohort. A significant cohort effect is evident. Nonetheless, this cohort effect may be attributable (at least in part) to other differences such as age, sex and education rather than depression. Chronic pain is positively associated with age but despite the PISA cohort being older on average (**Supplementary Figure 1**), the AGDS cohort showed a higher prevalence of chronic pain. After adjusting for all the relevant factors, the cohort effect was found to be partly attributable to depression status (OR = 1.86 [1.37–2.54]) because residual cohort effects were non-significant after accounting for the effect of depression ($Cohort_{AGDS}$ OR = 1.32 [0.97–1.79]). Furthermore, a higher age (OR = 1.02 [1.02–1.03]), lower educational attainment (OR = 0.89 [0.86–0.91]), and being female (OR = 1.16 [1.07–1.25]) were associated with chronic pain in the pooled PISA and AGDS sample (**Supplementary Table 1**).

TABLE 1 | Chronic pain prevalence in AGDS and PISA cohorts.

	Cases	Controls
AGDS (depression cohort)		
Sample size N (%)	6,895 (60.6%)	4,475 (39.4%)
Female N (%)	5,215 (60%)	3,402 (40%)
Age mean (sd)*	45 (15.1)	40 (14.3)
PISA		
Sample size N (%)	1,248 (50%)	1,221 (50%)
Depression* N (%)	119 (64%)	68 (36%)
Female N (%)	882 (51%)	854 (49%)
Age mean (sd)	60 (6.8)	60 (6.9)

Cases: participants reporting chronic pain.
Controls: participants reporting no chronic pain.
**$p < 0.05$ two-sample t-test or χ^2 test.*

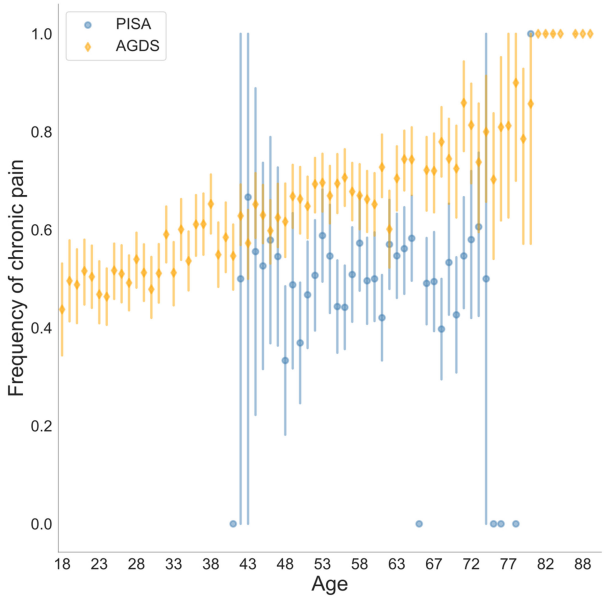

FIGURE 1 | Prevalence of chronic pain stratified by age and cohort (AGDS vs. PISA). Self-reported chronic pain was significantly higher in the AGDS cohort (N = 6,895/11,370) compared with the PISA cohort (N = 1,248/2,469)—OR = 1.31 (0.96–1.77); p = 0.086. For other statistical significance results see **Supplementary Table 1**. Both cohorts are population-based samples with AGDS being enriched for depression.

Chronic Pain Is Associated With Severity of Depression and Recent Suicidality

Results presented here are from AGDS where all participants reported depression. Higher pain severity (intensity) was found to be associated with longer durations of pain (**Supplementary Figure 3**). Increased pain severity was also associated with an increased number of depressive episodes (**Supplementary Figure 4**). The prevalence of suicidal ideation was higher in the comorbid chronic pain group (OR = 1.49 [1.38–1.61]). Likewise, recent suicide attempt was associated with chronic pain (OR = 1.88 [1.14–3.09]). Within the chronic

pain group, recent suicidal thoughts and suicide attempt scores were also positively correlated with chronic pain severity scores (**Supplementary Figure 4**).

Comorbid Psychiatric Diagnoses and Recent Substance Use

In this subsection, the results presented are from AGDS where all participants reported depression. Out of the 19 mental health conditions examined, social anxiety disorder was found to have the strongest association with chronic pain ($p < 0.01$), however, this association did not survive multiple-testing correction. Anorexia nervosa was found to be negatively associated with the likelihood of developing chronic pain ($p < 0.05$). Although both of these results were nominally significant, no association survived correction for multiple testing (**Figure 2**; **Supplementary Table 2**). Notably, chronic pain was significantly associated with decreased use of alcohol, increased use of tobacco, and painkiller misuse (e.g., opioids). Nominal associations were observed for other drugs such as cocaine (negative relationship) and opioids (**Figure 2**; **Supplementary Table 3**).

Chronic Pain and Antidepressant Response

In this subsection, the results presented are from AGDS, where all participants reported depression. The three most commonly prescribed antidepressants for the indication of chronic pain—over and above depression—were amitriptyline ($N = 606$), duloxetine ($N = 288$) and sertraline ($N = 160$; **Supplementary Figure 5**). Overall, the overwhelming majority of participants with chronic pain (i.e., 75–98% across all antidepressants) reported that their antidepressant prescription did not consider chronic pain. Furthermore, participants with chronic pain predominantly reported taking antidepressants for depression (i.e., more than 90% of participants reported their prescription was for depression). The exception was amitriptyline, for which only 60% of participants with chronic pain reported the prescription was for depression (**Supplementary Table 4**). Compared to participants without chronic pain, those with comorbid chronic pain were less likely to report positive functional benefits from taking antidepressants such as "*relief of depressive symptoms*," "*return of normal emotions*," and "*getting back to normal daily activities*" (**Figure 3A**). A trend was noted—whereby participants with chronic pain were more likely to report a *reduction in suicidal symptoms* as a positive aspect of antidepressant treatment—but this finding did not survive correction for multiple testing (**Supplementary Table 5**). Furthermore, in the chronic pain group, the average self-reported benefits from taking antidepressants were significantly lower compared to the group without chronic pain (OR = 0.75 [0.71–0.80]; $p < 2 \times 10^{-16}$). A similar but non-significant finding was observed between the average response of participants prescribed antidepressants for chronic pain vs. those without a chronic pain indication (OR = 0.94 [0.80–1.1]; **Supplementary Figure 6**). Next, we examined whether these findings held true for each antidepressant under investigation. For most antidepressants, no statistically significant difference in effectiveness was found between participants with chronic pain (or an indication for chronic pain) and participants without chronic pain. Participants with chronic pain who had taken sertraline, escitalopram or venlafaxine, reported significantly lower effectiveness than participants without chronic pain (**Figure 3B**). At this point, it remained unclear whether the antidepressant was taken before or after the commencement of chronic pain. To address this question, we performed a secondary analysis defining cases as participants who reported taking an antidepressant where the prescription explicitly considered chronic pain. This analysis revealed lower effectiveness for sertraline, escitalopram and citalopram (**Figure 3C**). The only antidepressants with a positive effect (i.e., greater effectiveness) were duloxetine, venlafaxine and amitriptyline, but only when prescribed for comorbid chronic pain and depression. However, none of these positive associations reached statistical significance (**Figure 3B**; **Supplementary Tables 6**, **7**).

DISCUSSION

We have reported the largest study to date on comorbid chronic pain and depression assessing the risk factors and treatment outcomes through comprehensive phenotyping of a depression-enriched sample. A key finding is that participants with comorbid chronic pain and depression reported significantly lower benefits from taking particular SSRI and SNRI antidepressants (i.e., sertraline, escitalopram, venlafaxine) compared to participants with depression but no chronic pain. Participants with comorbid chronic pain and depression also reported fewer functional benefits from taking antidepressants compared to those without chronic pain. For example, participants with comorbid chronic pain and depression treated with antidepressants were 22% less likely to report relief of depressive symptoms and 18% less likely to get back to normal daily activities compared to depression patients without comorbid chronic pain. The fewer functional benefits reported from antidepressants in those with comorbid chronic pain is consistent with prior research showing that pain is a strong predictor of non-remission with antidepressant medication treatment (83, 84).

We also found that participants prescribed particular SSRI antidepressants (i.e., sertraline, escitalopram, citalopram) *for chronic pain* reported significantly lower benefits (e.g., 55% lower odds of response from sertraline) compared to those taking the same medications but for a different indication. These results suggest that while SSRI and SNRI antidepressant classes may be equally effective in the treatment of comorbid chronic pain and depression (63, 85), specific antidepressants have differential effectiveness depending on certain common disease modifiers such as chronic pain. The lower effectiveness of sertraline is particularly important, as it is commonly used for the treatment of comorbid chronic pain and depression (60). Here we have shown evidence for differential effectiveness between several specific antidepressants in comorbid chronic pain and depression. We consider these findings to be robust because our methodological approach took into account the inherent clinical

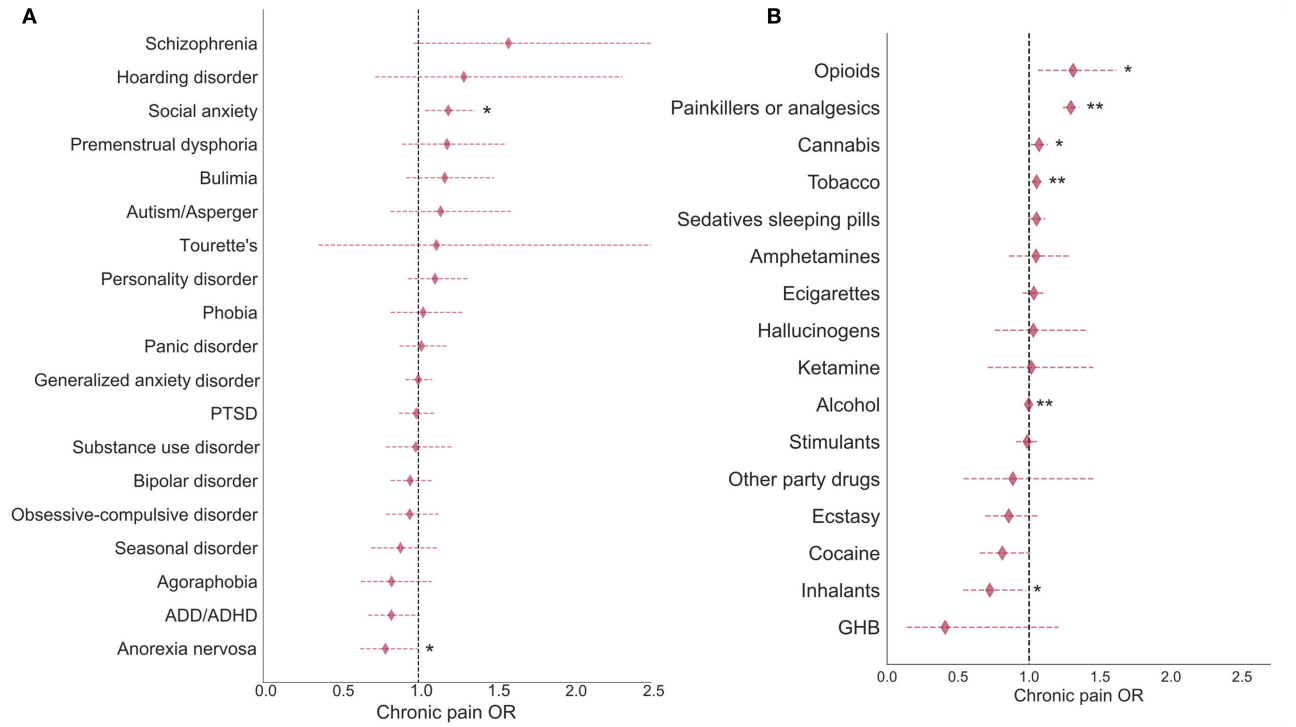

FIGURE 2 | Comorbidities and substance use associations with chronic pain in AGDS cohort. Forest plots depict the chronic pain odds ratios (ORs) for **(A)** comorbid disorders and **(B)** recent substance use during the past 3 months. Chronic pain was significantly associated with decreased use of alcohol, increased use of tobacco, and painkiller misuse (e.g., opioids). Diamonds represent ORs and horizontal lines depict 95% CI. ORs were estimated from a multivariate logistic regression accounting for all relevant covariates (see Methods). *$p < 0.05$; **$p < 0.05$ after Bonferroni correction for multiple testing (AGDS data only).

heterogeneity, high comorbidity and wide individual variation commonly observed in psychiatric disorders.

The current study's main findings of differential antidepressant effectiveness and fewer functional benefits from antidepressant use in comorbid chronic pain and depression are further underlined by demonstrating several results consistent with previous research. These include: (i) a strong association between depression and chronic pain (86, 87); (ii) increasing severity of chronic pain was associated with a higher number of depressive episodes experienced by participants (23); and (iii) older age, lower educational attainment and female sex were associated with higher chronic pain prevalence (3, 32–34, 88–90).

In the current study, amitriptyline was found to be the most commonly prescribed antidepressant to individuals with comorbid chronic pain. Indeed, it was prescribed over two times more than the next most commonly prescribed medication—duloxetine. Moreover, amitriptyline was the medication with, by far, the lowest indication for depression followed by duloxetine. Amitriptyline has traditionally been the first-line treatment for chronic neuropathic pain (61), however, its side-effect profile and mortality risk in overdose often limit its use (66). Our results are consistent with these medications being effective at treating chronic pain. In the current study, when the antidepressant prescription was for chronic pain—amitriptyline, duloxetine and venlafaxine showed a positive association with treatment effectiveness. This effect did not survive multiple-testing correction, which may be explained by the reduced power from further stratifying the sample. Our findings highlight the inadequate treatment recommendations for comorbid chronic pain and depression, as most participants with chronic pain did not report their antidepressant prescriptions were for chronic pain. The current study thus reaffirms the critical unmet need in this patient population.

While there have been conflicting reports regarding the link between chronic pain severity and suicidal behaviors (91–96), we provide evidence that supports an association between comorbid chronic pain and depression with both an increased risk for suicidal ideation and suicide attempt. Given suicide is a leading cause of death—particularly for young people (97)—and that depression and chronic pain are both treatable conditions, assessing their comorbidity in both at-risk youth and older adult populations may help to reduce suicide rates (98–101).

Consistent with previous observations (87), we also found comorbid chronic pain and depression was associated with recent increased use of tobacco and painkillers (e.g., opioids). However, we did not observe a significant association between comorbid chronic pain and depression with a self-reported substance use disorder. Previous reports suggest chronic pain,

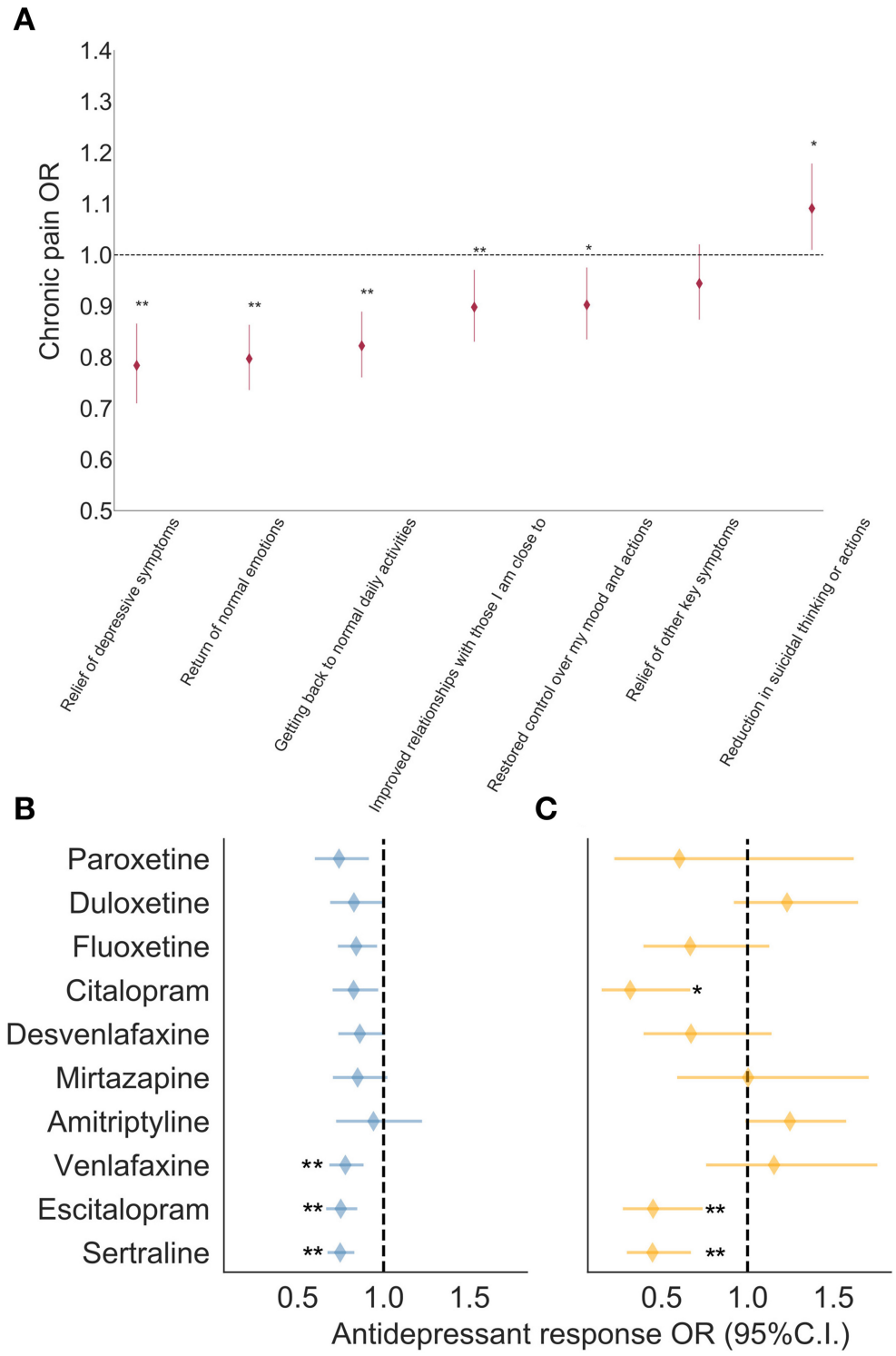

FIGURE 3 | Effect of chronic pain on antidepressant benefits in AGDS cohort. Forest plots depicting the results from: **(A)** association of chronic pain with self-reported benefits from general antidepressant treatment; and associations between antidepressant treatment response and **(B)** self-reported chronic pain or **(C)** self-reported prescription for chronic pain, while adjusting for the effects of sex and age at commencement of taking the antidepressant (*$p < 0.05$; **$p < 0.005$ statistical significance after Bonferroni correction for multiple testing; AGDS data only). Further details are in **Supplementary Figure 4** and **Supplementary Tables 4**, **5**.

depression and substance use disorder are often comorbid (102). It is possible that screening and diagnosis of substance use disorders in Australia may be lacking in those with comorbid chronic pain and depression. As such, clinicians need to consider substance use disorder in patients presenting with this comorbidity, as all three conditions increase the risk for other chronic diseases such as cardiovascular disease and cancer, while also increasing the risk of premature death (70).

Strengths, Limitations, and Further Research

The current study is the largest to date examining the relationship between depression and chronic pain with a novel pharmacoepidemiological approach that has yielded new insights into the medical treatment of these highly morbid conditions. However, there are also a number of limitations to be acknowledged. Data were primarily drawn from AGDS, which is a multi-aim study investigating the risk factors for depression and treatment response to antidepressants. As the AGDS employed phenotyping across an extensive range of complex traits (75), on balance it was not feasible to also collect detailed information from participants on dosages and (polypharmacy) combinations taken of the prescribed antidepressants (103); the duration and magnitude of benefits (e.g., for cost-effectiveness analyses) (65, 104, 105); drug tolerability and adverse events (65, 106–111); adjunct psychological therapies and multidisciplinary treatment/rehabilitation programs (112); other prescribed pain pharmacotherapies and questionnaires (113–117). Further pharmacoepidemiological studies focusing on chronic pain and a large range of psychiatric comorbidities (51, 52, 118). can directly address and collect data on these specific issues. As the current data were based on self-reported responses, they may also be subject to a degree of participant recall bias (e.g., time-specific details). For example, the substance use and suicidality phenotypes may be subject to non-disclosure effects due to the potential stigma associated with these conditions and the antidepressant response data may include non-specific (placebo/nocebo) effects (59, 119). However, these effects would be present across all antidepressants and thus alone are highly unlikely to explain the observed differences between the chronic pain (cases) and control participants. Randomized interventional studies comparing treatments for participants with comorbid chronic pain and depression are required to validate our results and elucidate the causal direction of associations reported here. Genetic-based methods can also aid in further examination of our findings by performing discovery genome-wide association studies (GWAS) of comorbid chronic pain and depression to determine individuals' polygenic risk irrespective of whether they have developed chronic pain or not. Furthermore, GWAS will enable: (i) the elucidation of causality between chronic pain and treatment response by using methods such as Mendelian randomization; and (ii) replication in antidepressant treatment-resistant depression cohorts with primary care and genotype data (120).

CONCLUSIONS

In summary, we found patients with comorbid depression and chronic pain were less likely to derive functional benefits from antidepressants (especially sertraline, escitalopram, and venlafaxine) than patients with depression but no chronic pain. Compared to patients with depression but no chronic pain, those with comorbid chronic pain and depression were also more likely to have had a recent suicide attempt, use tobacco and misuse painkillers. To further assess differences in effectiveness between specific antidepressants, targeted interventional trials can directly address the other phenotypes not captured in the current study. Nevertheless, our large-scale data-driven approach—like recent human genetic (36–52) and neuroimaging (57–59) antidepressant treatment studies—have revealed novel insights into the relationship between chronic pain and depression. Along with animal model and human pharmacogenetic studies (53–56, 116, 117, 121–125), there is also independent converging evidence for the critical role of subcortical brain regions in mediating pain and mood (126). The application of rigorous statistical genetics methodologies to large-scale neuroimaging data, for example, has already produced several major discoveries, such as advancing our understanding of causal pathways (subcortical), brain networks and medication response markers in mood disorders (127–133). Our study suggests pharmacoepidemiological approaches in psychiatry and pain medicine research will be increasingly valuable as a cost-effective, first-line strategy to enhance the design, feasibility, and clinical utility of randomized controlled trials (134), particularly as they routinely exclude patients with specific comorbidities and thus are not representative of the inherent individual variation across the population (104, 107, 135). Finally, the current study also has important implications for Australia's mental health system and chronic disease policy reforms, such as addressing problems concerning medication overprescription and effectiveness, their side-effects and suicide prevention (136–138).

DATA AVAILABILITY STATEMENT

The datasets generated for this article are not readily available because Access to the data in this study, even in anonymized formats is restricted due to ethical considerations. Access to the dataset can be granted only after review and approval by the QIMR Berghofer Human and Research Ethics Committee as well as the studies' private investigators. Requests to access the datasets should be directed to nick.martin@qimrberghofer.edu.au.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by QIMR Berghofer Medical Research Institute's Human Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WR, AC, TN, and MR designed this study and wrote the first version of the manuscript. AC performed the analyses with input from MR, TN, and WR. NM, SM, NW, and IH designed and directed the AGDS data collection efforts. NM and ML led the PISA study data collection efforts. TN designed the pain module in both the AGDS & PISA online surveys and conceived the genetic & epidemiological investigation of comorbid pain & depression in these cohorts. LG-M and GC-P contributed to data analyses. All authors contributed to the interpretation of the results and provided feedback on the preliminary versions 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/fpsyt.2021.643609/full#supplementary-material

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Conflict of Interest: IH has been Commissioner of Australia's National Mental Health Commission (2012–2018); Co-director of Health and Policy at the Brain and Mind Centre, University of Sydney; leading community-based and pharmaceutical industry-supported projects (Wyeth, Eli Lilly, Servier, Pfizer, AstraZeneca) focused on the identification and better management of anxiety and depression; a member of the Medical Advisory Panel for Medibank Private until October 2017; a board member of Psychosis Australia Trust; a member of the Veterans Mental Health Clinical Reference Group; and Chief Scientific Advisor to and an equity shareholder in Innowell. GC-P contributed to this study while employed at The University of Queensland. He is now an employee of 23andMe Inc and he may hold stock or stock options from the company.

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Actor and Partner Effects of Touch: Touch-Induced Stress Alleviation Is Influenced by Perceived Relationship Quality of the Couple

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Because of the impact of close partner's touch on psychological and physical well-being by alleviating stress, it is important to explore the influence factors that underlie the stress-alleviating effect of close partner's touch. Previous studies suggested that the stress-alleviating effect was different when individuals were touched by different persons. Specifically, the stress was reduced significantly when the individual was touched by the close partner compared with the acquaintance and the stranger. However, whether the stress-alleviating effect of touch was modulated by the close relationship quality is unknown. To examine this question, the participants (n = 61) performed a 3 (i.e., alone, partner no-touch, and partner touch) × 2 (i.e., safety and threat) within-subjects experiment. The results revealed that the stress of the participants alleviated significantly while close partners present with touch compared with without touch during facing a threat. We also found that the relationship quality of couple-members (i.e., participants perceiving the quality of alternatives and the partners' commitment level) modulated touch-induced stress alleviation. Participants perceiving the low quality of alternatives and the high partners' commitment level showed stronger touch-induced stress-alleviating effect than participants perceiving the high quality of alternatives and the low partners' commitment level. The explained variance was around 16.8% jointly for actor and partner effects. These findings provide evidence for explaining the reasons for touch-induced alleviating stress and have important implications for predicting the future effect of interactive behaviors.

Keywords: stress, relationship quality, touch, actor and partner effects, well-being

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INTRODUCTION

It is important to alleviate the negative impact of stress on people's health because a higher risk of diseases is linked to stress (Cohen et al., 2012). A previous research indicates that affectionate touch may buffer the potential negative impact of stressful events (Jakubiak and Feeney, 2019). Touch can convey different social intentions and messages, such as support, affiliation, dependence, and

hostility (Hertenstein et al., 2006a). Touch also has important social and affective values (Löken and Olausson, 2010), especially in close relationships (Jakubiak and Feeney, 2017). It has been shown that receiving positive affectionate touch promotes the development of secure attachment, interpersonal relationships, and psychological and physical well-being (Gallace and Spence, 2010; Devine et al., 2020). Given the powerful consequence of touch for psychological and physical health, researchers have long been interested in exploring how touch alleviates stress. However, relatively few studies have focused on the predictors of the touch-induced stress-alleviating effect.

Relationship quality is a complex and multifaceted concept with far-reaching social implications (Finkel et al., 2017). In the most general sense, relationship quality refers to the subjective perception about the negative or positive relationship statue (Beckmeyer et al., 2018). A growing number of research studies attempt to explain and predict relationship quality, and there have been many self-report predictors of relationship quality (e.g., attachment style, commitment, support, alternative, satisfaction) (Joel et al., 2020). Compared with other predictors, commitment and perceived quality of available alternatives are the powerful predictors of the stability of a romantic relationship (Kelley and Thibaut, 1978; Rusbult et al., 2001), and partner's commitment may be the important factor influencing individual perceiving of relationship stability. Hence, the commitment level of the partner and perceived quality of available alternatives were used to assess romantic relationship quality in the current study. The low quality of people's close relationships was associated with bad emotion (Cano et al., 2004), poor physical (Bookwala, 2005) and psychological health (Robles et al., 2014), and meant a decrease in individual resources. According to the Conservation of Resources (COR) theory, a decrease of resources related to the decline of close relationship quality triggers stress that can cause many negative outcomes (Hobfoll, 2001). Previous studies suggested that the stress-alleviating effect was different when individuals were touched by different persons who provided them with different resources. Compared with the acquaintance and the stranger, the stress was reduced significantly when the individual was touched by close partners (Goldstein et al., 2018; Morriss et al., 2019). It seems reasonable to assume that the higher the relationship quality of the couple, the stronger touch-induced the stress-alleviating effect will be, in both the individual and the partner. However, whether the touch-induced stress-alleviating effect is connected with the relationship quality of the couple remains unknown. Specifically, it is not clear whether individuals who perceive high relationship quality have stronger stress alleviation effect and, importantly, whether the partners perceive high relationship quality at the same time.

Here, we proposed two hypotheses, and we first hypothesized that the participants would report greater declines in stress when they received an affectionate touch from their close partners than when they received no touch (Hypothesis 1). Moreover, we hypothesized that touch-induced stress alleviation would be related to the relationship quality of couple-members and the relationship quality of both sides predicted the effect of touch-induced stress alleviation (Hypothesis 2). To test these hypotheses, 61 participants performed a 3 (i.e., alone, partner no-touch, and partner touch) × 2 (i.e., safety and threat) within-subjects experiment with their partners. By comparing the stress alleviation under partner touch condition while facing a threat with the stress alleviation under partner no-touch condition while facing a threat, the current study examined the role of close partners' touch in alleviating stress. Through multiple linear regression, we further examined whether the touch-induced stress-alleviating effect was modulated by the relationship quality of couple-members.

MATERIALS AND METHODS

Participants

The participants were recruited from the universities through advertisements posted on the campus Bulletin Board System (BBS). The participants were told that the study was about pain, and that they would go to the laboratory with their close partners for about twice. They were given no information about the effect of affectionate touch.

In previous research studies, compared with the acquaintance and the stranger, close partner's affectionate touch had a significant effect in relieving stress or pain while facing a threat (Coan et al., 2006, 2017). According to the average effect size of previous studies (effect size Cohen's $f = 0.5$), G-power was used to determine the appropriate sample size (effect size Cohen's f estimated at 0.50 to achieve 80% power, the required sample size was 18). To improve the reliability of the results, 64 participants (32 males and 32 females) took part in the experiment, aged from 18 to 25 years ($M_{age} = 21.6$ years, $SD_{age} = 1.4$ years). Three participants (two females and one male) were dropped from the analysis because the recordings of their self-report were always the same in the experiments.

The participants were screened by the following criteria: (i) right-handedness, (ii) no history of neurological disorders and mental illnesses, (iii) no medication used, (iv) no chronic or acute pain, (v) not pregnant, and (vi) in a heterosexual romantic relationship lasting more than 6 months. The partners in the experiment were their close partners. The study protocol was approved by the Ethics Committee of the University of Science and Technology of China. The written consents were signed by the participants, and every participant was paid 100 yuan for participation.

Procedure

During the first visit, the eligible participants came to the laboratory with their close partners. The experimenter introduced the tasks to them. Then, couple-members were assigned to different rooms to complete the questionnaire survey [demographic questionnaire and the Investment Model Scale (IMS)]. After finishing the questionnaire survey, the participants and their partners underwent pain familiarization and pain calibration, respectively. The participants received electric shocks to the left dorsal lower arm, each administered for 20 ms in ascending or descending order with 10 s interval. The participants were asked to report the pain intensity with the numerical pain scale (NPS), ranging from 0 to 100, denoting "no pain" to "the worst pain imaginable." At last, the stimulus

intensity, which evokes a pain magnitude of 70/100 (pain-70) on the NPS for three times, was chosen for every stimuli-target. Pain-70 intensities ranged from 3.0 to 3.8 mA, with an average of 3.41 mA and *SD* of 0.23.

Then, the participants and their partners came to the laboratory again to do the formal experiment after 2 or 3 days. They were brought to the experimental room and were asked to express their feelings and emotions naturally and not to talk with each other during the experiment. The participants who were subjected to the threat of electric shock (stimuli-targets) performed a 3 (i.e., alone, partner no-touch, and partner touch) × 2 (i.e., safety and threat) within-subjects experiment. The participants were instructed to rate their agitation and unpleasantness of the anticipation stage with an 11-point scale at every trial. Concurrently, their partners were asked to focus on the participants and rate the participants' level of unpleasantness and agitation. Both the participants and their partners rated the feelings in their non-dominant hand (left hands), and the right hands were used for affectionate touch (Goldstein et al., 2018). The difference with previous studies that highlighted touch-induced analgesia effects is the ratings of unpleasantness (agitation) about the anticipation stage, instead of the perception stage. Stress arises when one appraises a situation as a threat or demand on him and does not have adequate coping resource (Cohen and Wills, 1985). Because of the uncertainty of the threat cue (indicating a 25% likelihood of receiving an electric simulation), the electric shocks in the study were presented as the forthcoming threat. The study focuses on the stress, not the pain, so these ratings were all about the perceptions in the anticipation stage.

Experimental Conditions

The experiment included three conditions (alone, partner no-touch, and partner touch), each lasting about 12 min. A 10 min break separated every condition. During alone condition, the participants who were the stimuli-targets did the experiment in the room alone, holding the handles of the armchair by their dominant hands; meanwhile, their partners sat in another room. In partner touch condition, the participants and the partners sat face-to-face with hands-holding. While during partner no-touch condition, the participants and the partners sat face-to-face without hands-holding.

Each condition consisted of 24 trials. The trails were randomized within participants, and the condition order was counterbalanced between participants. The trial began with a threat cue or a safety cue (12 threat cues and 12 safety cues were presented in random order). Threat cue was a red "X" on a black background, indicating a 25% likelihood of receiving an electric stimulation to the left arm. Safety cue, which indicated no shock, was a blue "O" against a black background. The cue lasted for 1 s and was followed by a fixation cross, indicating a 4–10 s anticipation phase. At the end of the anticipation phase, electric stimulation might be delivered. Electric stimulation was produced by a separate physiological stimulator (Custom design, the maximum output voltage was +/−18 V, the peak-to-peak current range was adjustable continuously from 0 to 4 mA). Electric stimulation lasted for 20 ms at the current of pain-70 intensity. The ending cue of the anticipation phase was a dot. After that, the participants who were the stimuli-targets rated subjective perception of agitation (arousal) and unpleasantness (valence) during the anticipation phase by an 11-point scale. Meanwhile, the partners rated the level of agitation and unpleasantness of the stimuli-targets. The rating phase lasted for 12 s. At the end of each trial, the resting phase was presented with a black screen, varying from 4 to 10 s (**Figure 1**). All the participants received three or four shocks every condition (Coan et al., 2006).

Measures

Arousal and Valence

The couples were instructed to choose the rating numbers on the 11-point scale to rate the level of agitation and unpleasantness during the anticipation phase. The participants who were stimuli-targets rated their agitation (i.e., arousal) and unpleasantness (i.e., valence). In the meantime, the partners rated the level of stimuli-targets' agitation and unpleasantness according to their perception. The 11-point scale for agitation (unpleasantness) was rated with "calm" ("not unpleasant") at the left and "agitation" ("the most unpleasant imaginable") at the right (from 0 to 10). The purpose of asking the partners to focus on the stimuli-targets and rate the stimuli-targets' perception was to ensure that they were attentive. Only the agitation (unpleasantness) ratings of the stimuli-targets were analyzed in the current study.

Self-Report Psychometric Measure

In addition to the demographic information, all participants and their partners completed the IMS (Rusbult et al., 1998). The IMS includes four subscales (i.e., Satisfaction, Quality of Alternatives, Investment Size, and Commitment), with seven items for Commitment and five items for three other subscales. Satisfaction measures the degree to which a person is pleased with the close relationship ("My relationship is much better than other's relationships," "I feel satisfied with our relationship."); Quality of Alternatives measures the degree to which the partner can be replaced by someone attractive ("My needs for intimacy, companionship, *etc*., could easily be fulfilled," "If I were not dating my partner, I would do fine-I would find another appealing."); Investment Size measures the degree of personal involvement in the intimate relationship ("I have put a great deal into our relationship that I would lose," "I feel very involved in our relationship-like I have put a great deal into it."). Moreover, Commitment level measures the extent to which a person is committed to developing the intimate relationship ("I am committed to maintaining my relationship with my partner," "I am oriented toward the long-term future of my relationships."). The participants and their partners indicated their responses on a 9-point Likert-type scale, ranging from 0 = strongly disagree to 8 = strongly agree. The higher scores indicated higher levels of Satisfaction ($\alpha = 0.83$), Quality of Alternatives ($\alpha = 0.81$), Investment Size ($\alpha = 0.74$), and Commitment level ($\alpha = 0.78$), respectively.

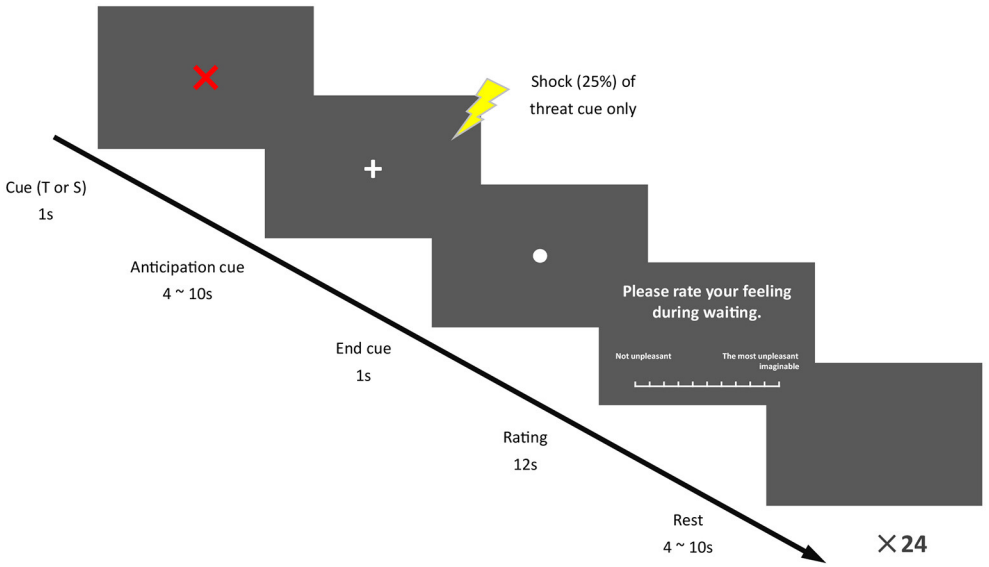

FIGURE 1 | Experimental procedure. Trials were composed of five phases, 1 s T or S cue, 4–10 s anticipation phase, 1 s end cue, 12 s rating phase, and 4–10 s resting phase.

Measure of Stress-Alleviating Effect and Arousal Reduction in No-Touch Condition and Touch Condition

The participants' ratings of unpleasantness (arousal ratings) in alone condition were set as the baseline. The stress alleviation (the arousal reduction) for partner touch condition was calculated as the difference between the participants' ratings of unpleasantness (arousal ratings) in alone condition and the ratings in partner touch condition. The stress-alleviating effect (arousal reduction) for partner no-touch condition was calculated as the difference between the participants' ratings of unpleasantness (arousal) in alone condition and the ratings in partner no-touch condition. The bigger the difference of the ratings was, the stronger the stress-alleviating effect (the reduction of arousal) was.

Touch-Induced Stress-Alleviating Effect

Touch-induced stress-alleviating effect was the difference between the participants' ratings of unpleasantness in partner no-touch condition and the ratings in partner touch condition. The bigger the difference between the ratings was, the stronger the touch-induced stress-alleviating effect was.

Statistical Analysis

Statistical analyses were carried out with the SPSS software (version 25.0). The continuous variables of interest were normally distributed, no transformations were necessary. We assessed the effect of partner touch on the stress alleviation and the arousal reduction by conducting a cue-type (threat, safety) × state (partner touch, partner no-touch) repeated measures ANOVA. Main effects and simple effects were tested *via* repeated measures ANOVA and paired *t*-test. Effect sizes were presented as partial eta-squared (η^2 partial) and *p*-value.

Pearson correlation was used to examine the correlation between perceived relationship quality of couple-members (participants perceiving the quality of alternatives and the partners' commitment level) and touch-induced stress alleviation. Multiple linear regression was used to explore the important indicators in influencing touch-induced stress-alleviating effect. The level of significance was set at $p < 0.05$.

RESULTS

Stress-Alleviating Effect of the Partner's Touch

We operated a series of repeated measures ANOVA to test our hypothesis. To examine the role of close partner's touch in alleviating stress, a 2 (cue-type: threat, safety) × 2 (state: partner touch, partner no-touch) repeated measures ANOVA on the stress-alleviating effect was conducted. As expected, the main effects of cue-type were found, $F_{(1,60)} = 4.61$, $p < 0.05$, $\eta_p^2 = 0.07$, and the stress-alleviating effect under threat ($M_{threat} = 0.54$, $SE = 0.18$) is significantly higher than that under safety ($M_{safety} = 0.11$, $SE = 0.09$, $t = 0.437$, $p < 0.05$). The main effects of state were significant, $F_{(1,60)} = 8.83$, $p < 0.01$, $\eta_p^2 = 0.13$, and the stress-alleviating effect of partner touch ($M_{partnertouch} = 0.49$, $SE = 0.13$) is significantly higher than that of partner no-touch ($M_{partnerno-touch} = 0.16$, $SE = 0.09$, $t = 0.323$, $p < 0.01$). There was also a significant cue-type × state interaction, $F_{(1,60)} = 9.56$, $p < 0.01$, $\eta_p^2 = 0.14$ (see **Figure 2**). The result of simple effects revealed that the participants who received touch from the partners ($M_{partner\ touch} = 0.83$, $SE = 0.24$) have stronger stress-alleviating effect than the participants who received no touch ($M_{partner\ no-touch} = 0.26$, $SE = 0.16$, $t = 3.47$,

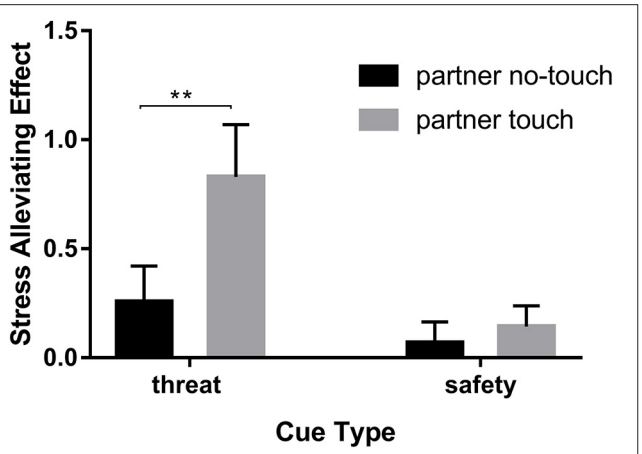

FIGURE 2 | The stress-alleviating effect of the partner's touch. $^{**}p < 0.01$.

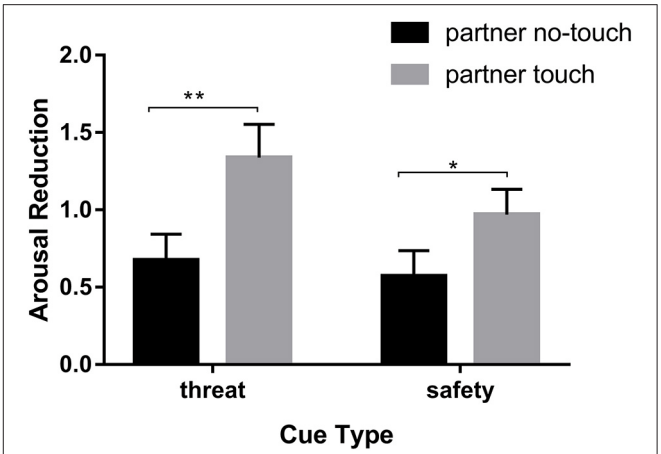

FIGURE 3 | Arousal reduction of the partner's touch. $^{*}p < 0.05$, $^{**}p < 0.01$.

$p < 0.01$) when facing a threat. When facing safety, the stress-alleviating effect was not different between two states ($M_{\text{partner no-touch}} = 0.07$, $SE = 0.09$; $M_{\text{partner touch}} = 0.14$, $SE = 0.09$, $t = -0.76$, $p > 0.05$). The interaction may have been driven by the significantly higher stress-alleviating effect for partner touch state than partner no-touch state while facing a threat, confirming that affectionate touch of close partner promotes stress-alleviating effect effectively.

Arousal Reduction of the Partner's Touch

A 2 (cue-type: threat, safety) × 2 (state: partner touch, partner no-touch) repeated measures ANOVA on arousal reduction was conducted for the purpose of investigating the arousal reduction of the partner's touch. The main effects of state were not significant, $F_{(1,60)} = 1.71$, $p > 0.05$, $\eta_p^2 = 0.028$. The main effects of cue-type were significant, $F_{(1,60)} = 8.98$, $p < 0.01$, $\eta_p^2 = 0.13$, and arousal reduction under threat ($M_{\text{threat}} = 1.15$, $SE = 0.16$) was significantly higher than that under safety ($M_{\text{safety}} = 0.62$, $SE = 0.14$, $t = 0.531$, $p < 0.01$). There was a marginal significant cue-type × state interaction, $F_{(1,60)} = 3.46$, $p = 0.068$, $\eta_p^2 = 0.054$ (see **Figure 3**). The result showed that the touch-induced arousal reduction effect was stronger while facing a threat. This indicated that emotional valence (unpleasantness) was more sensitive for indicating emotion than emotional arousal (agitation).

Perceived Relationship Quality of the Couple Modulating Touch-Induced Stress-Alleviating Effect

We hypothesized that touch-induced stress-alleviating effect would be associated with the relationship quality of the couples, with high relationship quality predicting greater touch-induced stress alleviation. A significant negative correlation was found between participants perceiving the quality of alternatives and touch-induced stress alleviation ($r = -0.266$, $p < 0.05$). A significant positive correlation was found between partner's commitment and touch-induced stress alleviation ($r = 0.324$, $p < 0.05$) (see **Figures 4**, **5**). Multiple linear regression was used to explore the degree to which perceived relationship of the couple-members (participants perceiving the quality of alternatives and the partner's commitment level) predicted touch-induced stress-alleviating effect.

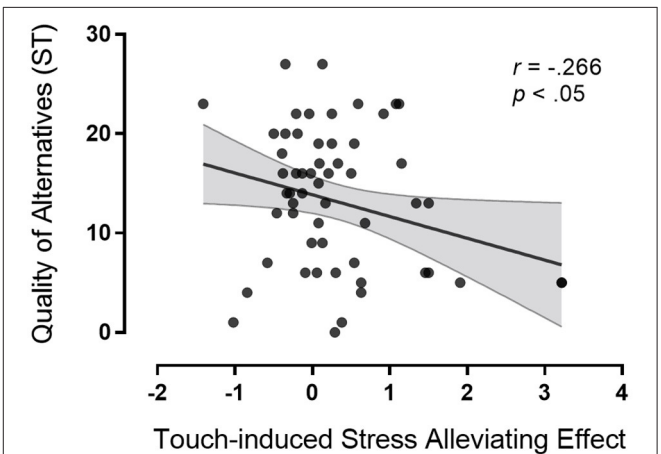

FIGURE 4 | Pearson correlation and scatterplots for perceived quality of alternatives and touch-induced stress-alleviating effect.

We found that the perceived relationship quality of the couple-members (participants perceiving the quality of alternatives and the partner's commitment level) modulated touch-induced stress alleviation. Participants perceiving the low quality of alternatives and the high partners' commitment level showed stronger touch-induced stress-alleviating effect than participants perceiving the high quality of alternatives and the low partners' commitment level. The findings indicated that the stress-alleviating effect of touch was affected by actor and partner effects. The explained variance was around 16.8% jointly for actor and partner effects ($R^2 = 16.8\%$, see **Table 1**). Moreover, we found that the stress-alleviating effect of the presence of the partner with no touch was only correlated with participants perceiving the quality of alternatives ($r = -0.285$,

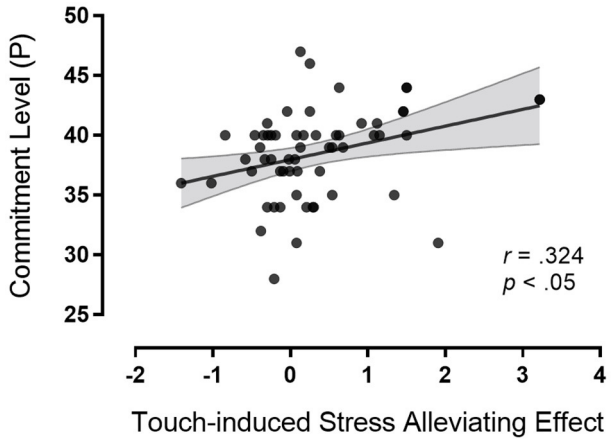

FIGURE 5 | Pearson correlation and scatterplots for commitment level of the partner and touch-induced stress-alleviating effect.

TABLE 1 | Relationship quality of the couples modulating touch-induced stress-alleviating effect.

	Partner's touch-induced stress-alleviating effect	
Predictor	**B**	***P***
Commitment level (P)	0.072 [0.017, 0.128]	0.012*
Quality of Alternatives (ST)	−0.031 [−0.060, 0.004]	0.038*

ST, stimuli-target; P, partner; B, unstandardized beta weights. 95% confidence intervals for significant effects are shown in brackets.
**p < 0.05.*

$p < 0.05$). Compared with participants perceiving the high quality of alternatives, participants perceiving the low quality of alternatives showed stronger stress alleviation.

DISCUSSION

Whether the close partner's touch can serve as a strong resource to alleviate individual stress to the threat? Whether the touch-induced stress-alleviating effect is modulated by the relationship quality of the couples? What are the important factors of relationship quality influencing the effect of touch-induced stress alleviation? The study aimed to answer these questions by an electrical stimulation experiment. The results revealed that the close partner's touch could alleviate individual stress effectively. The relationship quality of the couple (participants perceiving the quality of alternatives and the partner's commitment level) could predict up to 16.8% of the variance in touch-induced stress alleviation.

The finding that the participants who received a touch from the partners had a stronger stress-alleviating effect than the participants who received no touch was consistent with existing studies. In these studies, the partners' touch had a significant stress alleviation effect. However, these studies focused primarily on comparing touch-induced stress-alleviating effect between different social relationships (Coan et al., 2017; Morriss et al., 2019). The current experiment extended the previous research studies by comparing touch-induced stress-alleviating effect in different qualities of romantic relationships. Here, we showed evidence supporting the stress-alleviating effect of partner's touch and an impact of relationship quality on the stress alleviation of touch. Specifically, we demonstrated that the stress-alleviating effect of touch was affected by actor and partner effects (i.e., participants perceiving the low quality of alternatives and the high partners' commitment level showed stronger touch-induced stress-alleviating effect than participants perceiving the high quality of alternatives and the low partners' commitment level). As a non-verbal straightforward interactive way in emotional communication (Gallace and Spence, 2010), affectionate touch may be a bridge sharing dyadic interpersonal interaction process. Exploring touch-induced stress-alleviating effect from a two-way perspective will contribute to our understanding of affection interaction in an intimate relationship.

It has been proposed that perceived partner commitment was one of the most reliable factors predicting relationship quality and stability of relationship (Joel et al., 2020). Consistent with this proposition, we showed that the commitment of the close partner predicted the effect of touch-induced stress alleviation, suggesting a potential essence of love. Partners' commitment might increase individuals' social resources and enhance individuals' perceived support, thus having more confidence while facing a threat.

Some researchers examined the influence of affectionate touch on individuals' well-being and social relationship (Hertenstein et al., 2006b; Kim et al., 2018). Recently, Jakubiak and Feeney proposed a theoretical mechanistic model that affectionate touch might promote positive changes of relationship cognition, which reduce stress indirectly by enhancing relational, psychological, and physical well-being (Jakubiak and Feeney, 2019). However, the theoretical model neglects the interaction between the relationship quality and the behavior of touch. On the one hand, positive touch can promote relationship quality; on the other hand, relationship quality can influence the outcome of touch. Thus, our findings extend the recent theory explaining the theoretical mechanistic model of the stress-alleviating effect of touch and improve the theoretical model to fully explain why touching relieves stress. Specifically, we observed that the relationship quality of couples modulated the touch-induced stress alleviation.

The results indicated that the touch of the partners made the participants feel more secure under threat condition than the presence of the partners with no touch. In a digital immersive virtual environment study, Kane et al. found that the presence of the attentive partner made the participant feel more secure in a threatening cliff-walking task than that of the inattentive partner (Kane et al., 2012). Combined with the two studies, it will be significant for future research to explore the psychological mechanisms of different ways of social interaction-induced stress-alleviating effects.

Although the current research makes theoretical and practical contributions, it has several limitations that need to be acknowledged. First, causality between the perception of the relationship quality of both partners and affectionate

touch-induced stress alleviation should be considered with caution because of the correlational design of the current study. Although the causal inferences are limited by the correlational design, exploring the stress-alleviating effect of close partner's touch from dualistic perspective has its benefits. It is worth noting that the current study offers evidence for the value of a dualistic perspective on social interaction research. Second, data analysis of the study relies on the participants' self-reports, although the rating methods are often used in emotion-related studies. Thus, the objective indicator should be used in further research. Third, the research is limited by the characteristic of the sample, and participants are relatively young and unmarried couples. Compared with unmarried couples, married couples may have the emotion-rich experience, so more work needs to be done to verify the external validity of the results.

Taking these limitations into consideration, the robust causality experiment should be conducted. Moreover, future research should test moderator variables other than the perceived relationship quality of couple-members from a dualistic perspective. As one of the most fundamental communication ways, touch is associated with many physical and psychological outcomes for the couple, such as daily stress (Burleson et al., 2007), mood (Ditzen et al., 2008), and relationship satisfaction (Gulledge et al., 2003). Therefore, it is important for researchers to explore the active influencing factors of touch-induced positive effect.

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 Ethics Committee of University of Science and Technology of China (Date: 2020-05-26/No. 2020KY78). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DL, HS, and XZ conceived and designed the study. LZ, RM, and YZ recruited the participants and collected the data. WG supervised the data collection. YP and WL analyzed the data. DL and HS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Reactive Astrocytes: Critical Players in the Development of Chronic Pain

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Chronic pain is associated with long term plasticity of nociceptive pathways in the central nervous system. Astrocytes can profoundly affect synaptic function and increasing evidence has highlighted how altered astrocyte activity may contribute to the pathogenesis of chronic pain. In response to injury, astrocytes undergo a shift in form and function known as reactive astrogliosis, which affects their release of cytokines and gliotransmitters. These neuromodulatory substances have been implicated in driving the persistent changes in central nociceptive activity. Astrocytes also release lactate which neurons can use to produce energy during synaptic plasticity. Furthermore, recent research has provided insight into lactate's emerging role as a signaling molecule in the central nervous system, which may be involved in directly modulating neuronal and astrocytic activity. In this review, we present evidence for the involvement of astrocyte-derived tumor necrosis factor alpha in pain-associated plasticity, in addition to research suggesting the potential involvement of gliotransmitters D-serine and adenosine-5′-triphosphate. We also discuss work implicating astrocyte-neuron metabolic coupling, and the possible role of lactate, which has been sparsely studied in the context of chronic pain, in supporting pathological changes in central nociceptive activity.

Keywords: chronic pain, plasticity, astrocytes, cytokine, TNFα, lactate, gliotransmission

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INTRODUCTION

Chronic pain is associated with long lasting structural and functional reorganization of nociceptive circuits in the spinal cord and brain (1, 2). Historically considered supportive cells, mounting evidence indicates that astrocytes are dynamic players in neuroplasticity, and astrocytes have become increasingly recognized as active players in synaptic changes associated with chronic pain states. Pathologies of the central nervous system (CNS) often involve reactive astrogliosis, a process whereby astrocytes undergo a shift in morphology and function (3, 4). Reactive astrogliosis is associated with astrocyte hypertrophy, upregulated expression of glial fibrillary acidic protein (GFAP), and altered gene expression (5). Repeatedly, studies utilizing various rodent models of chronic pain show that GFAP is upregulated in the spinal cord (6–11) and brain areas involved in processing the sensory and affective components of pain, including the anterior cingulate cortex (ACC) (12–16), somatosensory cortex (17), amygdala (18, 19), thalamus (20), and ventrolateral periaqueductal gray (21–24). Notably, inhibiting astrocyte activity in the spinal cord (25–27) and primary somatosensory cortex (17) has been shown to reduce pain hypersensitivity. Chronic pain is also often associated with depression and elevated anxiety, and astrocyte inhibitors administered into the ACC have been shown to alleviate anxiety and depression-related symptoms in rodents (13, 15). Moreover, recent research has provided evidence for glial activation in the spinal cord and brain of patients with various chronic pain syndromes (28–31), and enhanced astrocyte activation

has been observed in the spinal dorsal horn of HIV patients with chronic pain (32). Given the consistent theme of astrocyte activation, recent research has focused on investigating the role of reactive astrocytes in the pathogenesis of chronic pain. This brief review will cover recent literature identifying astrocyte-derived cytokines, gliotransmission, and altered astrocyte-neuron metabolic coupling as potential contributors to the persistently altered synaptic activity observed in chronic pain states.

CYTOKINES

Cytokines are important regulators of inflammatory responses, and the activity of several pro-inflammatory and anti-inflammatory cytokines within the peripheral and central nervous systems (PNS and CNS, respectively) have been found to correspond with chronic pain states (33). Astrocytes and microglia release and respond to cytokines and play a significant role in immune responses of the CNS. Substantial research has uncovered microglia-mediated cytokine activity in chronic pain states. For example, spinal microglia are activated in inflammatory and neuropathic pain models (34, 35), and are associated with the production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), IL-6, and interferon gamma (IFNγ) (34, 36). Additionally, specific microglia inhibitors can attenuate pain-related behaviors while significantly inhibiting the upregulation of pro-inflammatory cytokines (37, 38). But while microglia contribute to the pro-inflammatory phenotype in concert with astrocytic activity, the present review will focus specifically on astrocytes [for excellent reviews regarding microglia in chronic pain, see references (39) and (40)]. Numerous *in-vitro* observations show that stimuli ranging from lipopolysaccharide, metabolic and mechanical stress, and neurotropic viruses, stimulate astrocytic production and release of cytokines (41–45). These include, but are not limited to, TNFα, IL-1α, IL-1β, IL-6, IFNα, IFN-β, and IFNγ (41–45). Work done in post-mortem tissue from chronic pain patients has associated spinal astrocyte activation with production of inflammatory cytokines such as IL-1β and TNFα (32), while an *in vivo* pain model has shown that inhibiting astrocytes with the toxin L-α-aminoadipate can reduce IL-1β expression and mechanical allodynia (46). Notably, elevated levels of pro-inflammatory cytokines have been observed in the blood and cerebral spinal fluid of patients with chronic pain, and have been shown to positively correlate with subjective ratings of pain intensity (47, 48).

TNFα DIRECTLY MODULATES NOCICEPTIVE NEURONAL ACTIVITY

TNFα's pathogenicity is well-documented in the peripheral nervous system. Direct injection of TNFα into the sciatic nerve or acute application to the L4 dorsal root ganglion (DRG) induces signs of mechanical allodynia and thermal hyperalgesia, however symptoms appear to be short-lived with recovery occurring within a couple days (49–51). Chronic application of a pad soaked in TNFα to the L5 nerve root or chronic perfusion of the DRG resulted in symptoms persisting beyond 7 days (52, 53), suggesting a requirement for extended exposure in the periphery to initiate long lasting pain. TNFα perfusion at the DRG is also able to enhance pain symptoms associated with compression of the DRG (53).

In the central nervous system, chronic pain induction elevates TNFα levels in the dorsal horn of the spinal cord, which either coincide with or are temporally close to the onset of mechanical and thermal hypersensitivity (54–58). In the brain, the time course of TNFα elevations in chronic pain models varies between different regions. For example, TNFα levels are elevated in the locus coeruleus prior to the onset of chronic constriction injury induced thermal hyperalgesia, while rises and falls in hippocampal TNFα approximately correspond to symptom onset and dissipation, respectively (58, 59). In the ACC, TNFα expression increases shortly after the onset of spared nerve injury-induced mechanical allodynia (60).

Research has shown that TNFα is able to modulate synaptic activity by acting directly on neurons via tumor necrosis factor receptor 1 (TNFR1) (61, 62) (**Figure 1**). Notably, astrocyte derived TNFα increases AMPA receptor and decreases $GABA_A$ receptor surface expression in cultured hippocampal neurons leading to increases in frequency and amplitude of mini excitatory postsynaptic currents (mEPSCs), and decreases in amplitude of mini inhibitory postsynaptic currents (61–63). Similar observations have been made in slices from the ACC, where TNFα increases the amplitude of evoked EPSCs and mEPSC frequency (64). TNFα can also increase the probability of presynaptic neurotransmitter release (64), through mechanisms that may involve the cation channel TRPV1 (65, 66). This TNFα mediated increase in neuronal excitability may participate in homeostatic synaptic scaling resulting from depressed synaptic activity, a process which is reliant on astrocytic rather than neuronal production of TNFα (67). Later work in neuronal cultures argues that rather than simply shifting neurons toward increased excitation, TNFα may act to permit rather than drive synaptic plasticity (68). Indeed, bidirectional effects have been observed, whereby high concentrations of TNFα (1 ug/mL) has been shown to impair the induction of long-term potentiation (LTP), while low concentrations (1 ng/mL) facilitate LTP (69). Studies assessing cytokine concentrations in chronic pain patients have found TNFα levels ranging from a few pg/mL or less in cerebrospinal fluid (47, 70), up to approximately 50 pg/mL in blood (48, 71, 72). As these concentrations are far below the level at which TNFα was found to impair LTP, TNFα release from reactive astrocytes may be more likely to instead facilitate synaptic potentiation. Accordingly, animal models of chronic inflammatory pain have also shown persistent 1–3 pg/mg increases in TNFα above baseline in the ACC and basolateral amygdala, which were associated with enhanced synaptic transmission in these regions, suggesting a possible role of astrocyte-derived TNFα in pain-induced hyperactivity (64, 73).

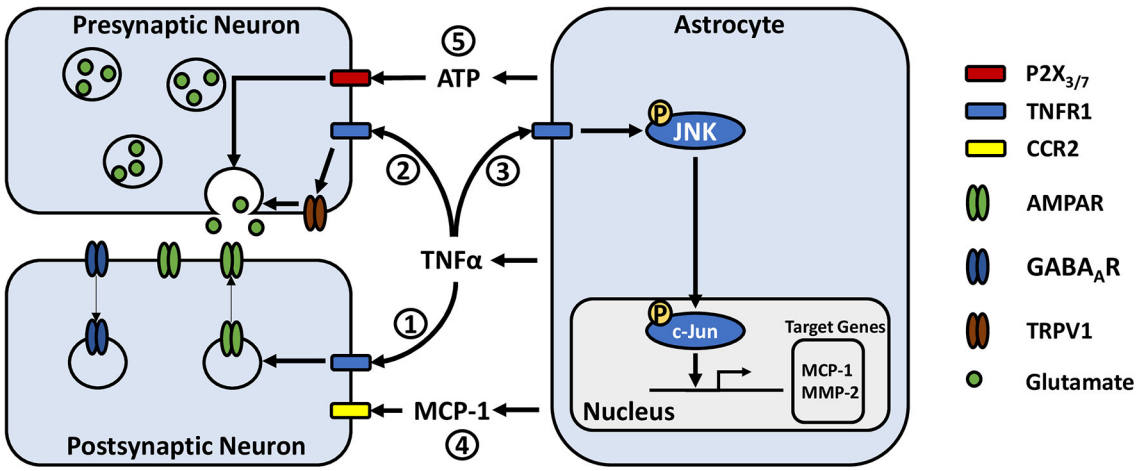

FIGURE 1 | Potential astrocyte-neuron signaling pathways in modulating pain-related synaptic transmission. (1) TNFα acts on neuronal TNFR1 resulting in rapid trafficking of GluR2-lacking AMPA receptors to the postsynaptic membrane and internalization of postsynaptic $GABA_A$ receptors. (2) TNFα increases presynaptic glutamate release, potentially by activating or increasing the expression of transient receptor potential subtype V1. (3) TNFα acts on astrocytic TNFR1, inducing phosphorylation of JNK. JNK phosphorylates c-Jun, which dimerizes with c-Fos to form the AP-1 transcription factor, leading to transcription of target genes such as MCP-1 and MMP-2. (4) MCP-1 is released from astrocytes where it can act on neurons via CCR2, modulating their excitability. (5) ATP released from astrocytes may act on pre-synaptic neuronal $P2X_3$ and $P2X_7$ receptors, stimulating glutamate release.

TNFα ACTIVATES THE JNK SIGNALING PATHWAY IN ASTROCYTES

Astrocyte-microglia crosstalk via cytokine signaling is emerging as an important mechanism in the development of chronic pain. Microglial release of IL-18 has been associated with astrocyte activation, while astrocytic CXCL12 has been reported to influence microglia, contributing to the development of mechanical allodynia in neuropathic and migraine pain models (74–76). Like neurons, both astrocytes and microglia express TNFR1 (77, 78) and are able to respond to TNFα (**Figure 1**). Recent cell culture studies have reported that activated microglia may induce reactive astrogliosis through the release of TNFα amongst other cytokines (79, 80). Additionally, astrocytic TNFα has been reported to exert autocrine effects in culture (81). A series of experiments by Yong Jing Gao and colleagues found evidence for a signaling pathway by which TNFα-induced astrocyte activation could contribute to persistent pain hypersensitivity. They showed in primary astrocyte cultures that TNFα induces transient TNFR1-dependent phosphorylation of c-Jun N-terminal kinase 1 (pJNK1) (82), a mitogen-activated protein kinase (MAPK). pJNK1 phosphorylates and activates c-Jun, which is part of the activator protein 1 transcription factor, leading to gene transcription (83). Activation of these astrocytes led to JNK1-dependent production and release of monocyte chemoattractant protein 1 (MCP-1) amongst other chemokines (82). Using *in vivo* approaches, they showed that TNFα injection into the mouse spinal cord induced JNK-dependent mechanical allodynia and thermal hyperalgesia at 3 h with a concomitant increase in MCP-1 expression in astrocytes (82, 84). Additionally, intrathecal injection of astrocytes incubated with TNFα were sufficient to induce MCP-1 dependent mechanical allodynia (84). These results are paralleled with their observations in a spinal nerve ligation model of neuropathic pain, where astrocytic MCP-1 is upregulated, and that pain hypersensitivity is significantly reduced by JNK inhibition and to a lesser degree by MCP-1 inhibition (82).

MCP-1 signaling via CC chemokine receptor type 2 (CCR2) modulates neuronal excitability. In culture, MCP-1 significantly reduces the responsiveness of neurons to GABA, as shown by a decrease in GABA-induced inward currents mediated by $GABA_A$ receptors (85). MCP-1 also causes significant increases in parameters indicating neuronal hyper-excitability, including decreased action potential current and voltage thresholds, as well as an increase in number of evoked action potentials (86). In spinal cord slice preparations, MCP-1 bath application dose-dependently enhances the frequency and amplitude of spontaneous EPSCs while enhancing inward AMPA and *N*-methyl-D-aspartate (NMDA) induced currents (82). Given these findings, persistent elevation of MCP-1 by TNFα provides a mechanism by which nociceptive sensitization may be maintained, as a constant shift toward excitation may reduce the threshold for initiating neuronal activity.

In addition to the induction of MCP-1, CCR2, which is constitutively expressed in the spinal dorsal horn (85), is upregulated in chronic pain states further amplifying the effects of MCP-1. In the peripheral nervous system, chronic compression injury to DRG neurons elevates CCR2 expression, increasing their likelihood to depolarize in response to MCP-1 *in vivo* and in dissociated neuronal cultures (86, 87). In bone cancer and trigeminal neuropathic pain models, CCR2 protein expression increases significantly in neurons of the ipsilateral superficial dorsal horn and medullary dorsal horn respectively, coinciding with onset of pain hypersensitivity (88,

89). CCR2 is also elevated in the nucleus accumbens shell where it modulates both depressive and pain-related symptoms (90), and the periaqueductal gray and rostral ventromedial medulla (24) which are involved in modulation of spinal nociceptive pathways (91).

It is likely that MCP-1 is not solely responsible for the nociceptive effects of JNK signaling, as MCP-1 inhibition only partially reduces pain hypersensitivity when compared to JNK inhibition (82). Matrix metalloproteinase-2 (MMP-2) and MMP-9, enzymes involved in degradation of the extracellular matrix [reviewed by Murphy, Nagase (92)], are also regulated by JNK signaling and secreted from astrocytes (93–95), and have been implicated in chronic pain. Work done in the hippocampus, classically associated with learning and memory rather than pain, has found that MMP-9 proteolytic activity contributes to maintenance but not induction of LTP (96), and MMP-9 alone is sufficient to enhance excitatory postsynaptic potentials and dendritic spine volume in CA1 neurons (97). In chronic pain however, while MMP-9 is upregulated in the peripheral nervous system, its expression in the CNS is minimal (98) and appears to be derived from DRG neurons rather than from astrocytes (99).

In contrast to MMP-9, delayed upregulation of MMP-2 is observed in spinal astrocytes following spinal nerve ligation, reaching significance 10 days after surgery (99). MMP-2 is sufficient to induce mechanical allodynia and is associated with cleavage of IL-1β, a major pro-inflammatory cytokine involved in neuroinflammation which works synergistically with TNFα. IL-1β can stimulate additional release of MMP-2 by activating extracellular signal-regulated kinase (ERK) 1/2 in astrocytes (99). Later work has identified the induction of neuronal MMP-2 at an earlier time point, coinciding approximately with symptom onset in a chronic post-ischemia pain model (100). Inhibiting MMP-2 reduces spinal GFAP levels along with decreased phosphorylation of JNK1/2 following induction of chronic pain (100). Thus, elevated neuronal MMP-2 may initially contribute to the induction of astrogliosis in the spinal cord, resulting in the phosphorylation of JNK in astrocytes, and release of both MMP-2 and MCP-1 during the chronic phase of pain. In line with this potential chain of events, there is some evidence suggesting a delay between onset of behavioral symptoms and elevated phosphorylation of JNK1. In two models of neuropathic pain, whereas behavioral symptoms manifested in under a day, increases in pJNK1 levels in the spinal dorsal horn were not observed until day 3 in a spinal nerve ligation model (101) or day 7 in a spared nerve injury model (102), suggesting a potential role for JNK1 in the transition from acute to chronic pain. Accordingly, in a CFA model of chronic inflammatory pain, whereas chronic intrathecal infusion of the JNK1 inhibitor D-JNKI-1 failed to reduce mechanical allodynia during the induction phase, tested at 6 h post CFA injection, it significantly reduced allodynia during the maintenance phase tested days 1–4 post CFA injection (103). In contrast, a study employing a mouse model of chronic post-ischemia pain observed elevations in pJNK1 at the same time as the development of behavioral symptoms (100), and JNK inhibition produced a significant analgesic effect at pain onset, indicating mechanistic differences in chronic pain development resulting from different injuries.

GLIOTRANSMISSION IN CHRONIC PAIN

Beyond their role in inflammatory signaling, astrocytes can detect neuronal activity through a variety of membrane receptors, inducing intracellular Ca^{2+} responses (104, 105) and subsequent release of neuromodulatory substances, known as gliotransmitters (106–108); these include glutamate, GABA, adenosine-5'-triphosphate (ATP), and D-serine, which bind to an array of pre- and post-synaptic neuronal receptors and influence synaptic transmission (109). The relevance of gliotransmission in chronic pain is currently under debate (110, 111), however emerging evidence indicates a potential role in chronic pain, including findings that gliotransmission is enhanced in reactive astrocytes (112–116) and modulated by inflammatory mediators (117, 118).

D-serine is a potent co-agonist which binds to the glycine site of NMDA receptors (119). It is synthesized by the enzyme serine racemase, which catalyzes the conversion of L-serine to D-serine (120). While serine racemase is primarily expressed in neurons (121), recent evidence shows that reactive astrocytes in traumatic brain injury, Alzheimer's disease, and pain models express the enzyme (115, 116, 122). D-serine released by reactive astrocytes is implicated in the expression of dynamic mechanical allodynia in chronic and acute models of orofacial pain, as well as static allodynia in chronic neuropathic pain (122–125). In these models, degradation of D-serine by D-amino acid oxidase or inhibition of serine racemase by L-serine O-sulfate can prevent the induction of mechanical allodynia, or reduce mechanical allodynia after onset (122–125). Despite neurons being capable of releasing D-serine (126), the finding that astrocyte inhibition reduces mechanical allodynia, which can be reversed by exogenous D-serine further suggests the specific requirement for astrocytes as a source of D-serine (124).

Another gliotransmitter that has been identified as a potential player in chronic pain is ATP. ATP acts primarily through the ionotropic P2X and metabotropic P2Y purinergic receptor families, which are expressed on many cell types in the CNS including neurons, astrocytes, and microglia [for a review see Burnstock (127)]. P2X receptor activation facilitates synaptic transmission by increasing presynaptic glutamate release (128, 129) and inducing EPSCs (130–132), while P2Y receptors primarily mediate inhibitory effects by reducing presynaptic glutamate release (129, 133). However, its functions at excitatory synapses are increasingly observed to be quite complex.

Work by Zhang et al. (134, 135) has provided evidence for the involvement of purinergic signaling on neurons in rats with chronic visceral hypersensitivity, showing that $P2X_7$ and $P2X_3$ are both upregulated and colocalize with the presynaptic marker synaptophysin in the insular cortex (134, 135). Additionally, inhibitors for either $P2X_7$ or $P2X_3$ reduced glutamatergic synaptic activity and pain-like symptoms, whereas agonists for either receptor had the opposite effect, elevating synaptic activity and inducing visceral hypersensitivity. Although the cellular source of ATP and whether it was enhanced was not tested in this model, studies by two separate groups in neuropathic pain models have found evidence suggesting that astrocytic ATP release in the spinal cord contributes

to pain hypersensitivity. One study by Cui et al. (136) found that inhibiting the mammalian target of rapamycin signaling pathway could reduce ATP release from cultured astrocytes and inhibit neuropathic pain-induced ATP elevation in cerebral spinal fluid. They associated this finding with the analgesic effect of rapamycin (136). Koyanagi et al. (137) investigated diurnal fluctuations in glucocorticoids and its effects on mechanical allodynia and observed that oscillations of plasma corticosterone levels corresponded to the oscillations of spinal ATP and mechanical allodynia. Intrathecal corticosterone injection induced mechanical allodynia which was dependent on microglial $P2Y_{12}$ and also induced ATP release from cultured astrocytes via serum/glucocorticoid regulated kinase 1 signaling (137). Here again like cytokine signaling, ATP signaling involves both astrocytes and microglia and represents another mechanism by which they can interact. Mixed glial culture studies have shown that activation of microglial P2 receptors by either exogenous or astrocyte-derived ATP induces the release of extracellular vesicles, which can in turn modify astrocyte activity (138, 139). Additionally, microglia can release ATP, which has been shown to indirectly modulate excitatory neuronal activity through binding to astrocytic $P2Y_1$ receptors in hippocampal slices (113). These findings provide a functional basis which may translate to pain-associated areas in the CNS. Indeed, studies in neuropathic pain models have associated the induction and activity of spinal microglia P2 receptors with the development of mechanical hypersensitivity (140, 141) [for a more in-depth discussion see review by Trang et al. (142)]. Given the variety of cells that can both release and respond to ATP, there is insufficient data to determine whether direct astrocyte to neuron purinergic signaling in the CNS is involved in chronic pain. However, due to the evidence for purinergic signaling in modulating synaptic activity, it remains a possible pathway by which astrocytes can influence maladaptive plasticity in nociceptive circuits of the CNS.

ALTERED GLUTAMATE-GLUTAMINE CYCLING IN CHRONIC PAIN

In addition to the release of neuromodulatory substances, astrocytes are metabolically coupled to neurons. They participate in glutamate clearance by taking up glutamate present at the synaptic cleft, which can then be converted to glutamine via glutamine synthetase (GS) and exported in a process known as the glutamate-glutamine cycle (143). Mounting human and animal data suggest that the development of chronic pain may also involve changes in glutamate-glutamine homeostasis. For example, in healthy human subjects, glutamate and glutamine levels positively correlate with subjective evoked pain ratings in pain-associated areas such as the ACC, mid-cingulate cortex, insula, dorsolateral prefrontal cortex, and thalamus (144, 145). Moreover, elevated combined levels of glutamate and glutamine have been observed in the ACC of patients with a range of chronic pain conditions (146), the thalamus of migraine patients (147), and in the right amygdala of female fibromyalgia patients (148). Notably, glutamate uptake by astrocytes, which is mediated via the glutamate transporters GLT-1 and GLAST (149), appears to show biphasic alterations in rodent models of neuropathic pain. Specifically, within the first 5 days following nerve injury, astrocytic expression of both in GLT-1 and GLAST is upregulated in the ipsilateral spinal dorsal horn (150, 151), which is followed by a prominent decrease in expression below baseline at 7 days post-injury and beyond (150–153). Critically, changes in glutamate uptake may play a causal role in chronic pain development, as inhibiting glutamate transporter upregulation enhances the onset and magnitude of pain-related behaviors (151), whereas transgenic upregulation of spinal GLT-1 can disrupt the induction of, and partially reverse, mechanical and thermal hypersensitivity in neuropathic and inflammatory pain models (154, 155). Interestingly, upregulation of GLT-1 was associated with a decreased number of dorsal horn neurons expressing the immediate early gene ΔFosB, indicating a reduction in neuronal activity (155). Additionally, acute inhibition of spinal GS has also been found to transiently reduce mechanical allodynia in a rat model of chronic pulpitis (156). This was accompanied by a reduction in the enhanced response of wide dynamic range neurons, located in the medullary dorsal horn, to mechanical stimulation (156). There are some discrepancies in the literature, as it has been found for example that intracisternal injection of DL-threo-β-benzyloxyaspartate, an inhibitor of GLT-1, GLAST, and the neuronal glutamate transporter EAAC1, can reduce rather than enhance CFA-induced orofacial heat hyperalgesia (157). Despite this, the evidence suggests that glutamate-glutamine cycling is altered in chronic pain states. It should be noted however, that while GLT-1 and GLAST are predominantly expressed on astrocytes, microglia in the spinal cord and brainstem have been shown to express both transporters following peripheral nerve injury (152, 158) and likely participate in mediating aberrant glutamate dynamics.

THE ASTROCYTE-NEURON LACTATE SHUTTLE

Astrocyte-neuronal metabolic coupling may also play a critical role in the development of chronic pain. Pain-induced neuroplasticity within spinal and brain regions is believed to promote the transition from acute to chronic pain, and mounting evidence indicates that astrocytes provide neurons with energy in an activity-dependent manner. In particular, astrocytes are the primary sites of glycogen storage in the CNS (159). In response to neuronal activity, astrocytes can rapidly metabolize glycogen to lactate (160) and export it to neurons, where it is converted to pyruvate, and metabolized to ATP via the citric acid cycle and oxidative phosphorylation to serve as a source of energy (161–163). L-lactate derived from astrocytes has been investigated as an energy source for neurons, and altered lactate metabolism is associated with diseases such as Alzheimer's (164–166), epilepsy (167), multiple sclerosis (168, 169), and depression (170). Astrocyte metabolism can also be significantly modified by a variety of cytokines (171). Therefore, it is of

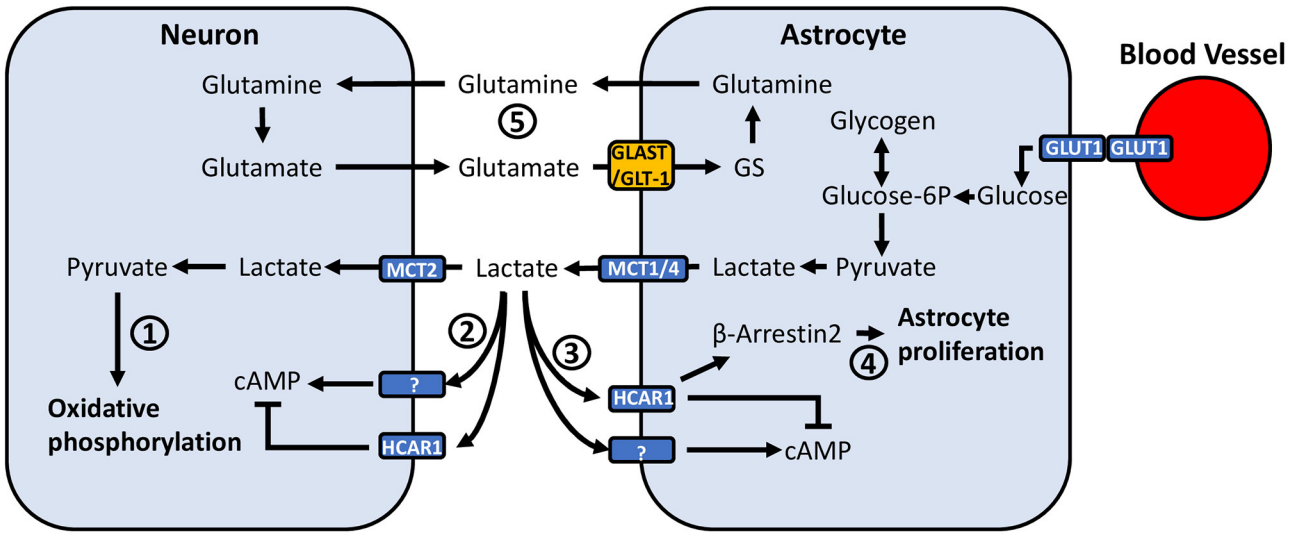

FIGURE 2 | Potential mechanisms through which astrocyte-neuronal metabolic coupling and lactate release can mediate pain-related neuronal activity. (1) Lactate taken up by neurons via MCT2 is converted to pyruvate, where it can be used to generate ATP through oxidative phosphorylation. (2) Neurons express HCAR1, a G_i-protein coupled receptor which inhibits adenylyl cyclase and reduces intracellular cAMP. Neurons also appear to express an unidentified lactate receptor which activates adenylyl cyclase and increases cAMP. (3) Similarly, astrocytes have been found to express HCAR1, however recent findings also suggest the presence of a lactate receptor which exerts opposite effects and activates rather than inhibits adenylyl cyclase. (4) Lactate binding to HCAR1 was recently associated with a non-G_i-protein mechanism, acting through β-arrestin2-MAPK-signaling. The β-arrestin2-MAPK pathway has been associated with induction of astrocyte proliferation. (5) Glutamate released into the synaptic cleft is taken up by astrocytes via GLT-1 and GLAST. Astrocytic GS converts glutamate to glutamine, which is exported and taken up by neurons. Altered GLT-1/GLAST expression may affect synaptic transmission through dysregulation of extracellular glutamate concentrations.

interest whether altered astrocytic lactate dynamics are involved in the development and maintenance of chronic pain.

Due to their close association with both neurons and blood vessels in the CNS, astrocytes are in a prime position to mediate energy supply to neurons (**Figure 2**). Astrocyte end feed processes make contact with blood vessels (172), allowing them to take up glucose from the blood via glucose transporter 1 (GLUT1) (173). Early culture studies demonstrated that astrocytes are able to accumulate glycogen in the presence of glucose (160). While both neurons and astrocytes express glycogen synthase (174, 175), a critical enzyme in glycogenesis, brain glycogen is predominantly localized to astrocytes rather than neurons (159). Astrocytes can break down glycogen to produce lactate (160), the majority of which is exported to the extracellular space rather than being consumed for energy (161, 162). In addition to findings that astrocytes can store glucose and export lactate, mechanisms by which lactate release is coupled to neuronal activity have been identified. Seminal work by Pellerin and Magistretti showed that glutamate uptake by astrocytes stimulates glucose uptake, glycolysis and lactate release, an effect dependent on extracellular Na+ and glutamate co-transport (176, 177). Further work identifying the expression of monocarboxylate transporters (MCT) in the brain, localization of different isoforms of lactate dehydrogenase in neurons and astrocytes, and that lactate was an efficient substrate for oxidative metabolism led to the proposal of the astrocyte neuron-lactate shuttle (178). This theory proposed that neuronal activity stimulates glycogenolysis and conversion of glucose to lactate, followed by its subsequent release from astrocytes and uptake by neurons to produce ATP during elevated activity (178).

Much of the work examining the consequences of lactate shuttling have been in the context of learning, memory, and reward associated learning in the brain. The formation of long-term memories has been shown to require hippocampal astrocytic lactate release during the initial acquisition period (179, 180). Indeed, training in an inhibitory avoidance task is accompanied by a rapid and sustained increase in extracellular lactate in the hippocampus (179), whereas inhibiting glycogen phosphorylase, the rate limiting enzyme in glycogenolysis, using 1,4-dideoxy-1,4,-imino-D-arabinitol 15 min prior to training abolishes the lactate rise, impairs LTP, and results in memory deficits (179, 180). In addition, inhibiting astrocytic lactate export by reducing MCT1 or MCT4 expression, or blocking neuronal lactate uptake by reducing MCT2 expression both result in memory impairments (179–181). The former can be rescued by lactate and its energetic equivalent pyruvate, but not by glucose (179, 180). Similar findings have been observed in drug-related memories involving the amygdala. In a conditioned place preference (CPP) paradigm, inhibiting glycogenolysis in the basolateral amygdala can prevent the acquisition of cocaine-induced place preference, transiently inhibit established CPP, and impair CPP following retrieval (182, 183). In both the hippocampus and amygdala, the induction of plasticity-related phosphorylation of cAMP response-element binding protein (CREB), cofilin, and ERK, is dependent on astrocytic glycogenolysis and L-lactate (179, 182). Notably, CREB

is activated in the spinal cord and forebrain following tissue injury (184, 185), and transgenic over-expression of CREB in the forebrain enhances behavioral responses to the formalin model of temporary pain, and correspond with potentiated and more rapid development of pain hypersensitivity induced by nerve injury (186). Given that long term memory and chronic pain both involve persistent changes in synaptic activity, it is of interest whether astrocyte-neuronal lactate shuttling is involved in the pathogenesis of chronic pain.

INITIAL RESEARCH ON ASTROCYTE LACTATE EXPORT IN CHRONIC PAIN

Very few studies have directly examined the involvement of astrocyte-derived lactate in the context of pain. However, there is some evidence associating chronic pain with altered lactate dynamics in the CNS. One study found that MCT1 protein expression is elevated in the spinal dorsal horn 7 days after induction of chronic inflammatory pain by CFA (187), an observation that was also made in the hippocampus following inhibitory avoidance training (179). Another study in rats with chronic visceral hypersensitivity observed blunted activity-dependent lactate release in the ACC along with impaired decision making and synaptic plasticity (16). Additionally, molecular changes associated with long-term potentiation, such as the upregulation of pCREB or pERK in the spinal dorsal horn and the spinothalamic tract (10, 188–192), as well as in supraspinal regions such as the amygdala and anterior cingulate cortex (193–195) are also observed in chronic pain states. These parallels with memory-related synaptic plasticity suggest the possibility of other common mechanisms such as lactate shuttling in chronic pain. Recent work by Miyamoto et al. (196) showed that activating spinal astrocytes in mice using designer receptors exclusively activated by designer drugs (DREADDs) rapidly induces mechanical allodynia lasting for 10 h, accompanied by an increase in extracellular lactate levels. Accordingly, the broad MCT inhibitor α-Cyano-4-hydroxycinnamic acid (4-CIN) fully reversed this induced allodynia (196). They also found that intrathecal injections of 4-CIN could reduce mechanical allodynia, although not fully, in a partial sciatic nerve ligation model of neuropathic pain (196). At the time of drug administration, behavioral symptoms have already developed suggesting that inhibiting lactate shuttling can reduce pain hypersensitivity during the chronic phase. A study by a separate group also found that 4-CIN could partially alleviate mechanical allodynia during the chronic phase of a spinal-nerve ligation pain model (197). Hence, this initial evidence points to a possible role of spinal astrocytic lactate in maintaining pain hypersensitivity. This is a departure from findings in long term memory, where disrupting lactate beyond a certain window of time following either memory acquisition or retrieval has no effect on subsequent task performance (179, 182).

In addition to the work above, there have been studies which investigated pyruvate kinase M2 (PKM2), a glycolytic enzyme that catalyzes the dephosphorylation of phosphoenolpyruvate to pyruvate. Expression of PKM2 is elevated in the spinal dorsal horn along with lactate in both neuropathic and inflammatory pain models (198, 199). Inhibiting PKM2 reduces lactate elevations and partially alleviates mechanical allodynia and thermal hyperalgesia (198, 199). However, these studies also noted that inhibiting PKM2 prevented the enhanced expression of GFAP, TNFα, IL-1β, and phosphorylation of STAT3 amongst other proteins (198, 199). Indeed, PKM2 has been implicated to have functions beyond glycolysis, including activity as a protein kinase [see review by Dong et al. (200)]. But these effects may also relate to possible lactate signaling which will be discussed below. It is however, difficult to isolate the PKM2-mediated rise in lactate to astrocytes in these studies, as PKM2 expression was elevated in neurons and microglia as well (199).

LACTATE SIGNALING ON NEURONAL EXCITABILITY AND PLASTICITY

Beyond acting as a metabolic substrate for ATP production, evidence for a signaling role of lactate complicates its effects in the CNS (**Figure 2**). The production of NADH by lactate in neurons has been shown to induce the expression of the plasticity-related immediate early genes *Arc*, *c-Fos* and *Zif268* (201). Additionally, neurons in the brain express the extracellular hydroxycarboxylic acid receptor 1 (HCAR1), a G_i-protein coupled receptor which inhibits adenylyl cyclase and reduces intracellular cAMP (202, 203). L-lactate has been found to decrease the firing frequency of CA1 pyramidal cells in hippocampal slices, as well as in primary cortical neuron cultures via activation of HCAR1 (203, 204). Conversely, L-lactate can potentiate EPSCs, firing frequency, and spike probability of pyramidal cells in the CA3 region of hippocampal slices (205), and similarly increase firing frequency and neurotransmitter release in locus coeruleus slices (206) via a lactate receptor that has yet to be characterized (205–207). These effects are suggested to be metabolism-independent and mediated by extracellular signaling due to insensitivity to 4-CIN (204–206). These findings raise the possibility that population differences in the effects of extracellular lactate signaling may result in differential regulation of synaptic activity at various points along nociceptive signaling pathways.

LACTATE SIGNALING IMPLICATED IN ALTERING ASTROCYTE FUNCTION

Astrocytes may also respond to lactate (**Figure 2**), as they express HCAR1 (202, 208), which was recently associated with reducing glutamate-induced calcium influx via β-arrestin2-MAPK signaling (209). Kappa-opioid receptor activation of the β-arrestin2-ERK1/2 pathway induces astrocyte proliferation *in vitro* (210), and kappa-opioid receptor activation of p38-MAPK, which is also regulated by β-arrestin2 (211), has been implicated in astrocyte proliferation following sciatic nerve ligation in mice (212). Thus, lactate may promote astrocyte proliferation via a common intracellular signaling pathway, contributing to reactive astrogliosis. Recent findings have also identified that lactate can induce rises in intracellular cAMP and lactate via activation of adenylyl cyclase, suggesting the presence of another

uncharacterized lactate receptor (213). cAMP is involved in a variety of signaling pathways and can modulate inducible nitric-oxide synthase activity (214, 215), cytokine release (216, 217), and astrocyte morphology (218).

Astrocytes in primary astroglial cultures incubated with 25 mM lactate show a significant increase in release of TNFα and IL-6 (219). Recent research in diabetic mice found that pyruvate dehydrogenase 2 (PDK2) expression was enhanced in hypothalamic astrocytes and contributed to inflammation (220). PDK2 phosphorylates and deactivates pyruvate dehydrogenase (221), shifting pyruvate metabolism to form lactate rather than enter the citric acid cycle. Genetically knocking out PDK2 reduced diabetes-induced elevation of lactate and induction of TNFα, IL-1β and IL-6, providing additional albeit indirect evidence for lactate-induced cytokine release (220). Furthermore, lactate uptake through MCT1 in oxygen and glucose deprived astrocyte cultures can upregulate expression of GFAP and phosphorylation of Akt and STAT3 (222); STAT3 is involved in reactive astrogliosis (223). These findings raise the possibility that aberrant lactate dynamics may facilitate astrocyte activation and their subsequent inflammatory cytokine profiles. However, given the expression of potentially two lactate receptors with opposing effects on adenylyl cyclase-cAMP signaling, the net effect of lactate on astrocytes in chronic pain is unclear.

Recent work by Bingul et al. (224) has provided interesting insight into long term lactate dynamics *in vivo* following LTP in the dentate gyrus of rats, with implications for lactate both as an energy substrate but also as a signaling molecule. Extracellular lactate levels change within seconds in response to acute electrical stimulation of the medial perforant pathway (224). The response is characterized by an initial dip in extracellular lactate followed by a larger overshoot, before returning to baseline (224). LTP induction causes a significant increase in the magnitude of the lactate dips and overshoots, starting at 24 h after potentiation, and induces an average chronic elevation of lactate concentrations that persists for 72 h (224). Whether these findings, in addition to lactate signaling described earlier, translates to CNS areas associated with mediating persistent pain has yet to be investigated, but they give rise to interesting possibilities. In the context of chronic pain, potentiated synaptic activity in the central nervous system may maintain persistently elevated extracellular lactate levels via activity-dependent release from astrocytes. Lactate, in addition to its metabolic role, is therefore in a position to mediate persistent effects on both astrocytes and neurons via extracellular receptor binding. However, lactate's functions as a signaling molecule in the CNS both under healthy and pathological conditions is poorly understood, requiring further research on how it may contribute to pathology.

CONCLUSION

Astrocytes release a variety of metabolites and cytokines which have profound effects on neuronal activity. Pathology of the CNS is often associated with reactive astrogliosis, which is accompanied by altered release of these neuromodulatory substances. The findings presented here provide evidence for the involvement of altered astrocytic cytokine release in long term synaptic plasticity of central nociceptive pathways under chronic pain states. Gliotransmitters have also been implicated but given that microglia are involved in ATP and D-serine signaling, whether direct astrocyte-neuronal communication via altered gliotransmission contributes to the pathology of chronic pain is unclear. Lastly, the role of lactate derived from astrocytes as a neuronal energy substrate, and more recently as a signaling molecule in the CNS, has evolved significantly. However, very few studies have examined the involvement of lactate in the development and maintenance of chronic pain, presenting an exciting pathway for further research.

AUTHOR CONTRIBUTIONS

JT and MB drafted the review. JT formatted **Figures 1**, **2**. JT and GD formulated the idea for the review and GD guided the research and writing process. All authors contributed to the article and approved the submitted version.

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Electroacupuncture Attenuates Anxiety-Like Behaviors in a Rat Model of Post-traumatic Stress Disorder: The Role of the Ventromedial Prefrontal Cortex

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Electroacupuncture (EA) is a promising clinical approach to treating posttraumatic stress disorder (PTSD), yet the mechanisms whereby EA can alleviate anxiety and other PTSD symptoms have yet to be clarified. In the present report, rats underwent EA for 14 consecutive days following modified single prolonged stress (MSPS) exposure. These animals were then evaluated in open field and elevated plus maze tests (OFT and EPM), while Fos immunohistochemical staining was performed to assess ventromedial prefrontal cortex (vmPFC) functional activation. In addition, an extracellular recording and stimulation system was used to analyze vmPFC inputs into the ventral tegmental area (VTA) in these rats. Temporary vmPFC inactivation was further performed to assess whether this was sufficient to reverse the anxiolytic effects of EA. Overall, rats that underwent EA treatment spent more time in the central region (OFT) and the open arm (EPM) relative to MSPS model animals ($P < 0.05$). These MSPS model animals also exhibited significantly fewer activated Fos-positive nuclei in the vmPFC following behavioral testing, while EA was associated with a significant relative increase in c-Fos expression in this region. The transient inactivation of the vmPFC was sufficient to reverse the effects of EA treatment on anxiety-like behaviors in MSPS model rats. MSPS and SEA rats exhibiting no differences in bursting activity between baseline and vmPFC stimulation, whereas bursting activity rose relative to baseline upon ventral mPFC stimulation in EA treated and control rats. Together, these findings indicate that the vmPFC and its inputs into the VTA are functionally linked to the anxiolytic activity of EA, implicating this pathway in the EA-mediated treatment of PTSD.

Keywords: posttraumatic stress disorder, electroacupuncture, anxiety-like behaviors, ventromedial prefrontal cortex, ventral tegmental area

INTRODUCTION

Posttraumatic stress disorder (PTSD) is a serious psychological condition that can arise in individuals who have experienced or witnessed a traumatic event, causing a range of symptoms including depression and anxiety that can adversely impact an affected individual's overall quality of life (Woodward et al., 2000). Both pharmaceutical- and psychotherapy-based approaches have been used to treat the symptoms of PTSD (Taylor et al., 2006). Acupuncture is a traditional Chinese medicinal practice that has been conducted for over 2,000 years, and that can alleviate stress-related anxiety and depression *via* the hypothalamic-pituitary-adrenal axis (HPA) (Grant et al., 2018; Oh et al., 2018). Hollifield et al. (2007) determined that PTSD patients undergoing acupuncture exhibited significant decreases in clinical symptoms comparable to those of patients undergoing cognitive-behavioral therapy. Experiments from our lab and others have shown that electroacupuncture (EA) can achieve anxiolytic activity in PTSD model rats (Oh et al., 2018; Li et al., 2019; Liu et al., 2019; Xue et al., 2019). The mechanisms whereby this form of acupuncture can treat anxiety-related behaviors associated with PTSD, however, remain to be fully clarified.

The ventromedial prefrontal cortex (vmPFC) is located in the medial prefrontal cortex, and plays central roles in regulating both goal-directed and anxiety-related behaviors (Kim et al., 2011; Arnsten et al., 2012; Padilla-Coreano et al., 2016). The pathophysiological basis of PTSD is thought to be partially attributable to reductions in vmPFC top-down emotional modulation (Nicholson et al., 2017). Abnormal apoptotic cell death within the vmPFC has recently been detected in the traditional single prolonged stress (SPS) model of PTSD (Jia et al., 2018; Pati et al., 2018), and SPS model animals also exhibit reductions in vmPFC c-Fos immunoreactivity (Yu et al., 2015; Pati et al., 2018). Furthermore, SPS can induce hypoactivity in the vmPFC and impaired prefrontal cortex control of amygdala and striatum in rats (Piggott et al., 2019). There is also a growing body of evidence suggesting that anxiety modulation and PTSD incidence are related to the dopamine (DA) system in the ventral tegmental area (VTA) (Corral-Frias et al., 2013; DeGroot et al., 2020). The inactivation of DA neurons within the VTA can reduce PTSD-like behavior incidence or intensity, in addition to significantly reducing baseline firing of these dopaminergic VTA cells (Corral-Frias et al., 2013). Furthermore, vmPFC inputs into the VTA play a key role in the functionality of this DA system and the associated regulation of anxiety (Knowland and Lim, 2018), with the vmPFC exhibiting direct innervation of DA neurons in the VTA (Gariano and Groves, 1988), controlling DA release within the nucleus accumbens (Taber et al., 1995; You et al., 1998). Some researchers have posited that vmPFC afferents to the VTA may serve as a key source of anxiety-related glutamate within the VTA (Felix-Ortiz et al., 2016). Recent studies have shown that acupuncture involves cortical modulation (Hauck et al., 2017). The anterior cingulate cortex, for example, is critical for the effects of EA on anxiety-associated behaviors in both SPS and formalin-induced pain rat model systems (Yi et al., 2011; Liu et al., 2019). Prior work from our lab has also shown that EA can alter vmPFC neuron firing activity (Zhang et al., 2017).

Herein, we utilized a rat SPS model system to explore the impact of EA on anxiety-like behaviors through its ability to influence vmPFC neurons and inputs into the VTA. Functional vmPFC activation following EA was assessed *via* Fos immunomapping, while behavioral experiments were used to assess anxiety-related behaviors. Temporary vmPFC inactivation was additionally conducted to confirm whether such inactivation was sufficient to reverse the anxiolytic effects of EA. In addition, extracellular recordings of anesthetized rats were employed to investigate whether PTSD was associated with vmPFC inputs into the VTA and whether these effects were reversed upon EA treatment.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley (SD) rats (220–250 g) from the Shanghai Experimental Animal Center were purchased and allowed to acclimate for 7 days prior to experimental use. Rats were housed in a controlled environment (25 ± 2°C, 12 h light/dark cycle) with free food and water access. This study was conducted as per NIH standard guidelines and received approval from the Shanghai University of Traditional Chinese Medicine Animal Care and Use Committee.

Modified Single Prolonged Stress

An MSPS model was established as reported previously (Su et al., 2013; Perrine et al., 2016), with modifications having been made in our laboratory (Liu et al., 2019). Briefly, rats were subjected to three consecutive stressors: restraint, forced swim, and anesthesia stresses. Restraint was achieved by placing rats into plexiglass cylinders for 2 h such that they experienced head immobilization. Rats were then placed in an acrylic cylindrical bucket (40 cm diameter, 50 cm height) that contained fresh water (20–25°C, two-thirds full) and forced to swim for 20 min. After drying and recovering for 15 min, rats were anesthetized to unconsciousness such that no response to tail or toe pinch was evident using pentobarbital sodium. Rats were then returned to their cages for 7 days.

EA Treatment

Electroacupuncture stimulation was conducted while rats were gently restrained. Sterile stainless steel needles (0.16 mm diameter, 13 mm long) were inserted bilaterally into acupoint ST36 between the anterior tibialis and extensor digitorum longus muscles proximal to the knee joint. Electric stimulation was generated by a stimulator instrument (Shanghai Medical Electronic Apparatus, China), and was delivered using two needles. The frequency of stimulation was 2 Hz. The intensity of the stimulation was increased stepwise from 0.5, 1.0 to 1.5 mA, with each step lasting for 10 min. Sham control rats had needles inserted into these ST36 acupoints but did not undergo electrical stimulation.

Behavioral Testing

Elevated Plus Maze Testing

An EPM apparatus (Shanghai Jiliang Software Technology Co., Ltd.) was used for this study. This maze was composed of two open arms facing in opposite directions and two closed arms facing in opposite directions that were 50 cm above the floor (all arms were 15 cm wide and 45 cm in diameter). As detailed previously (Liu et al., 2019), rats were placed in the center of the maze facing an open arm at the start of the test, and were allowed to roam freely for 15 min during which time a video tracking apparatus recorded their movement and behaviors with the EthoVision software (v7.1). Both the time spent in open arms and the percentage of time spent in open arms were measured as exploratory behaviors of interest, with an arm entry being recorded when a rat entered a given maze arm with all four paws.

Open Field Test Analyses

Open field test analyses are routinely used when measuring anxiety and spontaneous locomotor activity (Schmitt and Hiemke, 1998). Briefly, rats were placed in the central region of 40 cm × 40 cm × 50 cm apparatus that was separated into central and peripheral regions by gray lines. Rats were then allowed to freely roam through this apparatus for 15 min, during which time a video-tracking system (Shanghai XinRuan Information Technology Co., Ltd.) recorded their behavior, with the EthoVision software [v 7.1] being used to automatically analyze the time spent in the central region and the total distance covered by these rats.

c-Fos Immunohistochemical Staining

At 1.5 h post-behavioral testing, rats were deeply anesthetized using pentobarbital sodium (100 mg/kg, ip), sequentially perfused transcardially with saline (200 mL) and 4% paraformaldehyde (PFA) in 0.1 mol/L phosphate buffer (PB) (250 mL). Brains were then collected from each animal, fixed overnight in 4% PFA, and transferred to 30% sucrose for 5–7 days until the tissue was saturated and had sunk to the bottom of the solution. Samples were then sliced to yield a series of 30 μm-thick coronal sections using a chilled (−25°C) cryostat instrument, with the resultant sections being transferred to PBS. The staining of these sections for c-Fos was conducted as in prior studies (Georges et al., 2006). Briefly, sections were washed thrice with PBS, blocked for 2 h with 1% BSA at 4°C, and probed for 48 h with mouse monoclonal anti-c-Fos (1:400; sc-166940, Santa Cruz) at 4°C. Following three subsequent washes with PBS, sections were stained for 2 h with mouse IgGk light chain-binding protein (m-IgGk BP-PE, 1:200; sc-516141, Santa Cruz). Sections were then mounted on adhesive slides to which coverslips and fluorescence decay-resistant medium were applied. A Leica Laser Scanning Confocal Microscope was then used to analyze these stained sections. The best standard stereotaxic plane sections of the vmPFC were identified as per Paxinos and Watson's atlas (George and Charles, 2013), with numbers of c-Fox positive cells per section then being assessed (20×). An automatically generated 200 μm × 500 μm rectangle was used to denote the vmPFC in each section, and an analytical software was used to calculate the number of stained nuclei per section. Numbers of c-Fos-positive nuclei per section were thereby determined and averaged to yield representative results for analysis.

Pharmacological Inactivation

One week before EPM testing, rats (300–350 g) were intraperitoneally injected with pentobarbital sodium (50 mg/kg) and mounted on a stereotaxic frame, with isoflurane (1.5–2%) being delivered *via* a nosecone. Bilateral guide cannulae (26 gauge, Plastics One) were implanted in the vmPFC (+ 3.0 mm AP; ± 0.8 mm ML; –3.8 mm DV). The cannulae were fixed to the skull with dental cement and three steel screws. To ensure that the cannulae remained unobstructed, stainless steel obturators were inserted. In EA group, rats were cannulated after EA treatment. Rats were systemically treated with benzylpenicillin sodium (60,000 U) to prevent infection, and were allowed to recover for 5–7 days after surgery.

Five minutes prior to EPM testing, rats were bilaterally injected with 0.3 μl of artificial CSF (aCSF) or an equivalent volume of aCSF containing 1.0 nmol/0.1 nmol mixture of baclofen and muscimol (GABAB and GABAA receptor agonists, respectively; Tocris Bioscience). For these injections, 33-gauge injection cannulae were inserted into the guides and extended 1 mm below the guide cannula tip, with solutions being delivered through PE50 tubing *via* microinfusion pump over a 1 min period using a microsyringe. Following injection, cannulae were allowed to remain in place for 1 min, and were then removed to permit fluid diffusion.

Following behavioral testing, rats were euthanized *via* sodium pentobarbital overdose (100 mg/kg, ip), perfused sequentially with 0.9% saline and 4% PFA in PBS (250 mL), after which brains were collected, stored in 30% sucrose as above, frozen, and sliced with a cryostat to yield 40 μm sections. Cannula placement was confirmed by comparing cannula-related damage to reference images in a rat brain atlas (George and Charles, 2013). Due to cannula misplacement, one rat was excluded from behavioral testing analyses.

Stimulation and Recording

Stereotaxic Surgery

Initial anesthetization was achieved by placing rats in a closed container containing 2.5% isoflurane, after which a tracheotomy was conducted and a 1.0–1.5% isoflurane solution was delivered under spontaneous respiration *via* a tracheal cannula to maintain anesthetization during surgery. Rats were mounted in a stereotaxic apparatus and warmed to 36–38°C with a heating pad. The skull was then exposed, and holes above the vmPFC (2.8 mm rostral and 0.4–0.6 mm lateral to bregma) and the VTA (5.3 mm caudal and 0.5–0.8 lateral to bregma) were drilled.

Electrical Stimulation of the vmPFC

A bipolar concentric electrode (250 μm in diameter overall; 50 μm diameter for inner electrode) was inserted into the vmPFC (5.0 DV from skull surface). Electrical stimulation was then achieved using a square pulse stimulator controlled by the Spike2 program (CED 1401, Spike2; Cambridge Electronic

Design, Cambridge, United Kingdom) (0.5 Hz, 0.5 ms pulse duration). Two intensity levels (0.5 and 1.0 mA) were tested during stimulation.

VTA Recording

Recording in the VTA was achieved by inserting a glass micropipette (tip diameter, 1–3 μm; 6–12 MΩ) containing a solution of 2.0% pontamine sky blue solution in 0.5 M sodium acetate into the VTA (DV: 6.5–9.0 mm). Electrophysiological criteria detailed previously were used to identify spontaneously active DA neurons in this region (Tong et al., 1996; Georges et al., 2006; Ungless and Grace, 2012). DA neuron activities recorded within the VTA included: (1) action potentials (APs) exhibiting biphasic or triphasic waveforms > 2.5 ms in duration, (2) > 1.1 ms from spike onset to negative trough, and (3) a slow spontaneous firing rate [> 10 spikes/second (sp/s)]. Bursts were defined by the detection of two consecutive spikes with an interspike interval < 80 ms, whereas burst termination was defined by two spikes for which this interval was > 160 ms (Tong et al., 1996; Georges et al., 2006; Ungless and Grace, 2012; Kaufling and Aston-Jones, 2015). Signals were amplified and filtered (0.1–5 kHz bandpass) with standard electronic equipment. Single neuron spikes were identified and interpreted as digital pulses by the computer through the use of the Spike2 software. Upon isolating a single neuron, spontaneous baseline activity prior to stimulation was recorded for at least 5 min, after which 50 individual electrical pulses were delivered to the vmPFC, and the responses of these VTA DA neurons were recorded.

Histological Analysis

Electrode locations were verified after recording session completion. Sites of vmPFC stimulation were demarcated by applying 10–20 μA of positive current for 1–2 min through the stimulating electrode to generate a lesion, while sites of VTA recording were demarcated based upon the presence of iontophoretic pontamine sky blue deposits following the application of an alternating current (−7 μA) for 12–15 min. Rats were then euthanized, and brain tissue sections were prepared as above with recording sites being verified by Leica Microsystems.

Statistical Analysis

Data are means ± SEM. Behavioral and c-Fos staining data were analyzed *via* one-way ANOVAs with Fisher's *post hoc* LSD test where appropriate. $P < 0.05$ was the threshold of significance for these analyses.

For electrophysiological analyses, both firing rate and the percentage of spikes occurring in bursts (%SIB) were analyzed. Burst onset was defined by the detection of two spikes within < 80 ms of one another (Schmitt and Hiemke, 1998), while%SIB was calculated before and after vmPFC stimulation by dividing the total number of spikes that occurred in bursts by the total spike number over the measured time period. Excitatory and inhibitory epochs of VTA DA neurons in response to stimulation of the vmPFC were assessed as detailed previously (Jodo et al., 1998; Moorman and Aston-Jones, 2010). During vmPFC stimulation, cumulative VTA activity peri-stimulus time histograms (PSTHs) with a bin width of 5 ms were generated for all recorded neurons (Jodo et al., 1998). These PSTHs were used to assess response magnitude (Rmag) for excitation and inhibition, and were normalized to baseline spontaneous firing activity levels. Briefly, baseline average counts (per bin) were initially determined over the 500 ms period prior to stimulation, with excitation onset being the first five bins in which the mean value was greater than two standard deviations above mean baseline activity levels. Response offset was defined as the time when this activity had returned to levels within two standard deviations of the baseline mean values. Inhibition was defined as the presence of an epoch at least 15 bins in length during which the mean count per bin was at least 35% below baseline. The resultant data were given as means ± SEM, and were analyzed *via* one-way ANOVAs with Fisher's LSD test as appropriate. Chi-squared tests were used to compare relative proportions of inhibited or excited neurons in the vmPFC between treatment groups, with mean firing rate and %SIB values during vmPFC stimulation within each group were compared using paired *t*-tests.

RESULTS

EA Treatment Impacts MSPS-Related Anxiety-Like Behaviors

We began by using the OFT and EPM tests to evaluate the impact of EA treatment on anxiety-like behaviors in MSPS model rats. OFT and EPM test were conducted in the morning and afternoon, respectively, with at least 4 h between tests to ensure they did not interfere with one another. Rats were randomized into four groups ($n = 8$/group): control, MSPS, EA, and Sham EA (SEA) groups. MSPS group rats did not undergo EA, while control group rats did not undergo MSPS modeling. EA group rats were treated for 30 min/day for 14 consecutive days beginning 1 week after MSPS model establishment, while SEA rats underwent the same treatment regimen but without the application of electrical stimulation during EA therapy.

EA Alters MSPS Model Rat Performance in OFT Analyses

In OFT analyses, significant differences were detected among rats in the four different treatment groups with respect to the amount of time spent in the central region [$F(3,28) = 9.337$, $P < 0.001$], the distance traveled in the central region [$F(3,28) = 7.49$, $P < 0.001$], and entries into the central region [$F(3,28) = 8.753$, $P < 0.001$] (**Figure 1**). Through *post hoc* analyses, we determined that rats in the MSPS group exhibited significant reductions in time spent in the central region ($P < 0.001$), distance traveled in the central region ($P < 0.01$), and entries into the central region ($P < 0.001$) relative to control rats. Relative to rats in the MSPS model group, EA group rats spent significantly more time in the central region ($P < 0.01$), in addition to exhibiting increased walking distance in the central region ($P < 0.05$), and more

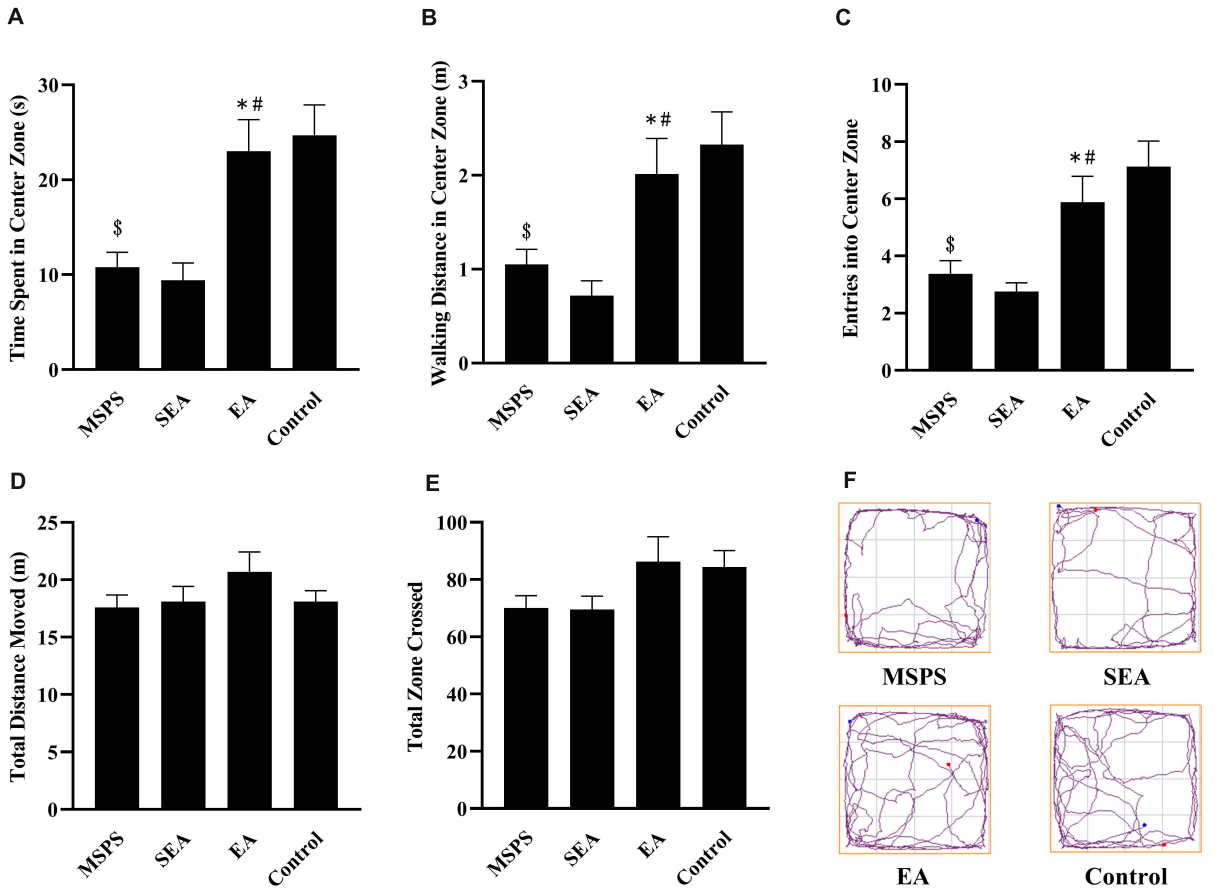

FIGURE 1 | EA at ST36 alters MSPS model rat anxiety-like behaviors in OFT experiments. **(A)** Time spent in the central region in seconds. **(B)** Distance traveled in the central region (m). **(C)** Central region entries. **(D)** Total distance moved (m). **(E)** Total zone crossings. **(F)** Representative tracking plots. Data are means ± S.E.M. ($^{\$}P < 0.05$, PTSD vs. control groups; $^{*}P < 0.05$, EA vs. SEA groups; $^{\#}P < 0.05$, EA vs. PTSD groups).

entries into the central region ($P < 0.05$). No such difference, in contrast, was observed for rats in the SEA group relative to the MSPS group. No differences were observed among groups with respect to zones crossed [$F(3,28) = 2.175$, $P > 0.05$] or total distance traveled [$F(3,28) = 1.163$, $P > 0.05$].

EA Alters MSPS Model Rat Performance During EPM Testing

Significant differences were detected *via* one-way ANOVA when comparing rats in the four treatment groups with respect to time spent in the open arms [$F(3,28) = 7.316$, $P < 0.001$], percentage of time spent in open arms [$F(3,28) = 7.317$, $P < 0.001$], and open arm entries [$F(3,28) = 7.008$, $P < 0.001$]. Rats in the MSPS group were less active in open arms than were control rats (**Figure 2D**), exhibiting significant decreases in the amount of time spent in open arms ($P < 0.01$; **Figure 2A**), the percentage of time spent in open arms ($P < 0.01$; **Figure 2B**), and open arm entries ($P < 0.01$; **Figure 2C**). Relative to these MSPS model animals, those in the EA group exhibited significant increases in time spent in open arms ($P < 0.01$), open arm entries ($P < 0.05$), and percentage of time spent in open arms ($P < 0.01$), whereas such differences were not observed when comparing rats in the MSPS and SEA groups ($P > 0.05$).

EA Treatment Alters vmPFC c-Fos Expression Following Behavioral Testing in MSPS Model Rats

The pathophysiological basis for PTSD is thought to be at least partially attributable to reduced top-down emotion modulation from vmPFC regions (Nicholson et al., 2017). To examine the link between the beneficial effects of EA on anxiety-like behaviors and vmPFC functional activation, c-Fos immunomapping was thus conducted. At 90 min post-behavioral testing, four rats per group were selected at random. Three consecutive sections were taken from each animal for IHC staining analyses of the vmPFC region. Significant differences in vmPFC c-Fos levels were observed among treatment groups [$F(3,44) = 7.19$, $P < 0.01$] (**Figure 3**). Specifically, there were significantly fewer Fos-positive neurons in the MSPS and SEA groups relative to the control group (**Figures 3a,c,d**), whereas these numbers were significantly higher in rats in the EA group relative to those in the MSPS and SEA groups ($P < 0.01$, **Figures 3a,e**). No differences between the

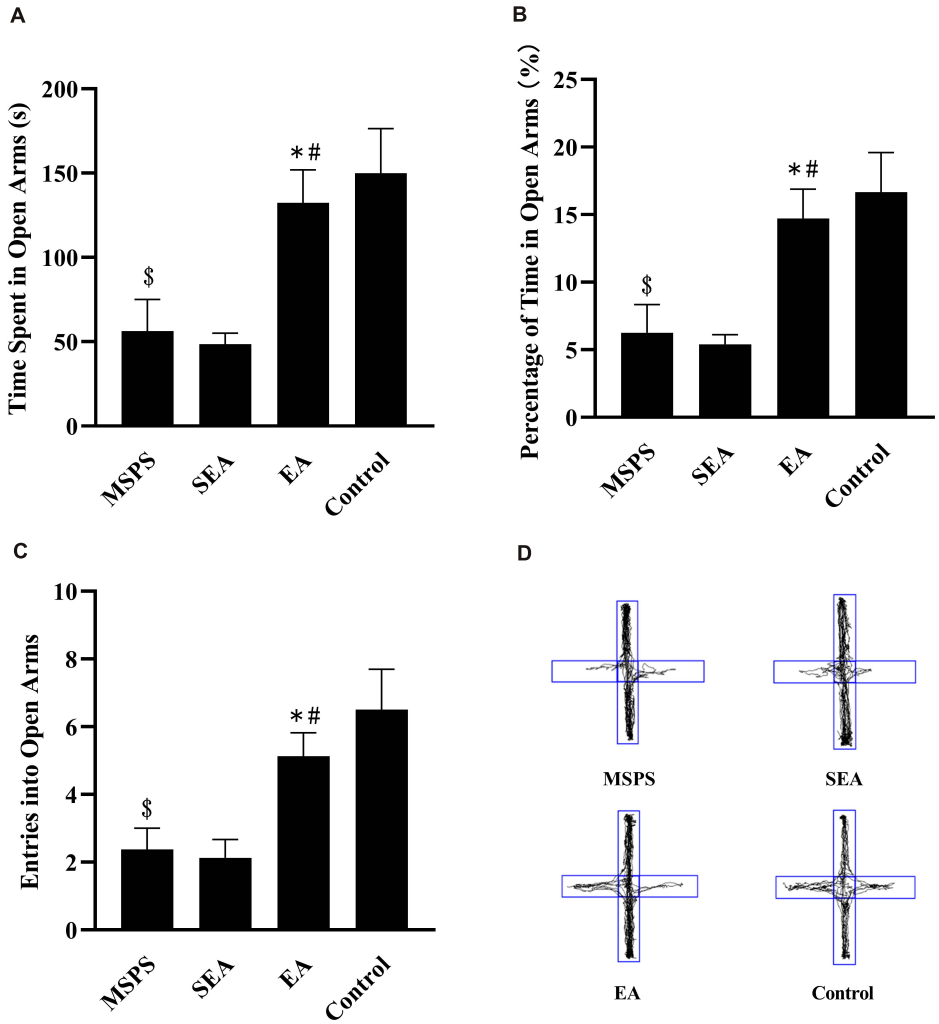

FIGURE 2 | Impacts of EA at ST36 on anxiety-like behaviors in MSPS rats as measured in an EPM. **(A)** Time spent in the open arms in seconds. **(B)** Percentage of time spent in open arms. **(C)** Open arm entries. **(D)** Representative tracking plots. Data are means ± S.E.M. ($^{\$}P < 0.05$, PTSD vs. control groups; $^{*}P < 0.05$, EA vs. SEA groups; $\#P < 0.05$, EA vs. PTSD groups).

EA and control groups were observed with respect to c-Fos expression in the vmPFC ($P > 0.05$, **Figures 3a,e,f**).

Transient vmPFC Inactivation Ablates the Effects of EA on Anxiety-Like Behaviors

To clarify the relationship between the vmPFC and the apparent anxiolytic responses associated with EA treatment in PTSD model rats, we next conducted transient vmPFC inactivation immediately before behavioral testing to see whether this was sufficient to reverse the anxiolytic responses observed following EA treatment (**Figure 4A**). One-way ANOVAs indicated that such treatment was associated with significant differences in time spent in open arms [$F(3,20) = 7.59$, $P < 0.001$] and the percentage of time spent in open arms [$F(3,20) = 8.33$, $P < 0.001$] among groups, with EA treatment having augmented the amount of time spent in open arms as above (MSPS + EA vs. MSPS + BM, $P < 0.05$, **Figures 4B,C,F**). Prior research has indicated that inactivating the vmPFC can result in increased anxiety as measured *via* EPM testing (de Visser et al., 2011; Pati et al., 2018). We similarly found that the observed increases in time spent in open arms during EPM testing in the EA group were ablated by transient vmPFC inactivation. Indeed, animals in the MSPS + EA + BM group exhibited significantly less time spent in the open arm during EPM analyses relative to rats in the MSPS + EA group (MSPS + EA vs. MSPS + EA + BM, $P < 0.05$, **Figures 4B–E,G**).

VTA Inputs From the vmPFC Are Impacted by MSPS and EA Treatment as Determined Through Electrophysiological Analyses

Ventral tegmental area inputs from the vmPFC play a key role in the overall function of the DA system, regulating its

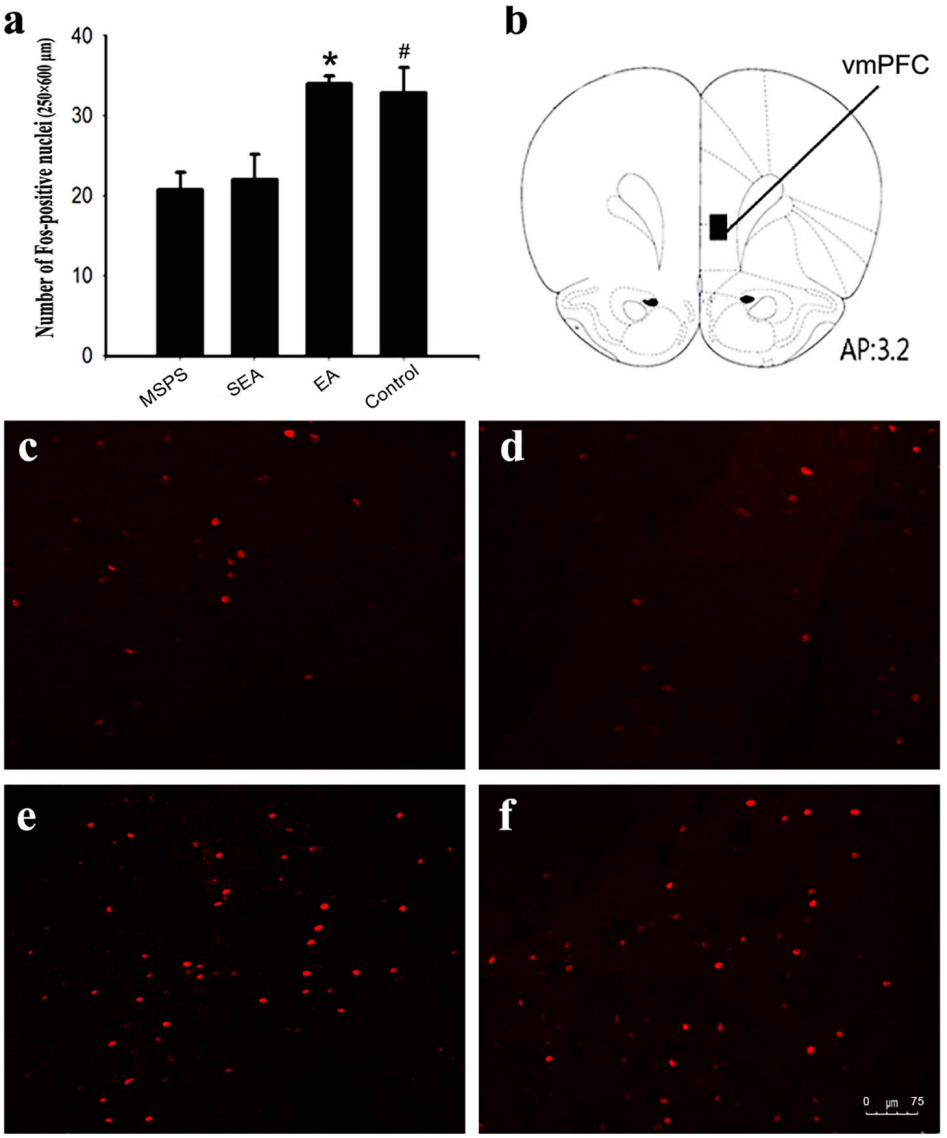

FIGURE 3 | EA treatment increases the number of Fos-positive nuclei in the vmPFC of MSPS model rats. **(a)** Quantitative assessment of Fos-positive nuclei in the vmPFC. Data are means ± SEM. *#$P < 0.05$ vs. PTSD group. **(b)** Schematic overview of a coronal section through the vmPFC, with a 200 μm × 500 μm rectangle highlighting the region of the vmPFC in which Fos-positive nuclei were counted. The number at the bottom of the schematic indicates the distance from the bregma in millimeters. This image was adapted from an atlas produced by Paxinos and Watson. **(c–f)** Representative coronal sections illustrating Fos staining in the vmPFC: **(c)** MSPS group; **(d)** SEA group; **(e)** EA group; **(f)** Control group.

influence on anxiety-like behaviors following trauma (Taber et al., 1995; Arnsten et al., 2012; Corral-Frias et al., 2013). To explore VTA DA neuron responses to vmPFC electrical stimulation in the context of MSPS modeling and EA treatment, we next conducted *in vivo* extracellular single-unit recording studies of 55 histologically verified DA neurons from 25 rats based on their exhibiting wide spikes (>2.5 ms) and a wide initial action potential (AP) component (Ungless and Grace, 2012). GABA-like neurons exhibiting rapid firing rates and think spikes were not a focus of the present study. An overview of the experimental workflow for these experiments is shown in **Figure 5A**, with stimulation sites being shown in **Figures 5B,C**, and with the plots of these 55 histologically localized DA neurons recorded in the VTA being shown in **Figure 5D**. All DA neurons recorded in this study exhibited an AP width > 1.1 ms (Ungless et al., 2004), and no differences were observed among groups with respect to the neurons meeting these criteria (**Figure 6A**) [ANOVA; $F(3,51) = 2.15$; $p > 0.05$]. VTA DA neurons in PTSD group rats exhibited a mean firing rate of 29.8 ± 5.5 spikes/10 s, while these rates were 24.9 ± 3.7 spikes/10 s, 24.8 ± 5.2 spikes/10 s, and 28.4 ± 3.9 spikes/10 s in SEA, EA, and control rats, respectively (**Figure 6B**). The percentage of spikes occurring in bursts (%SIB) in the PTSD, SEA, EA, and control groups was 31.2 ± 5.8%, 29.1 ± 4.2%, 34.2 ± 3.3%, and 35.3 ± 5.6%, respectively

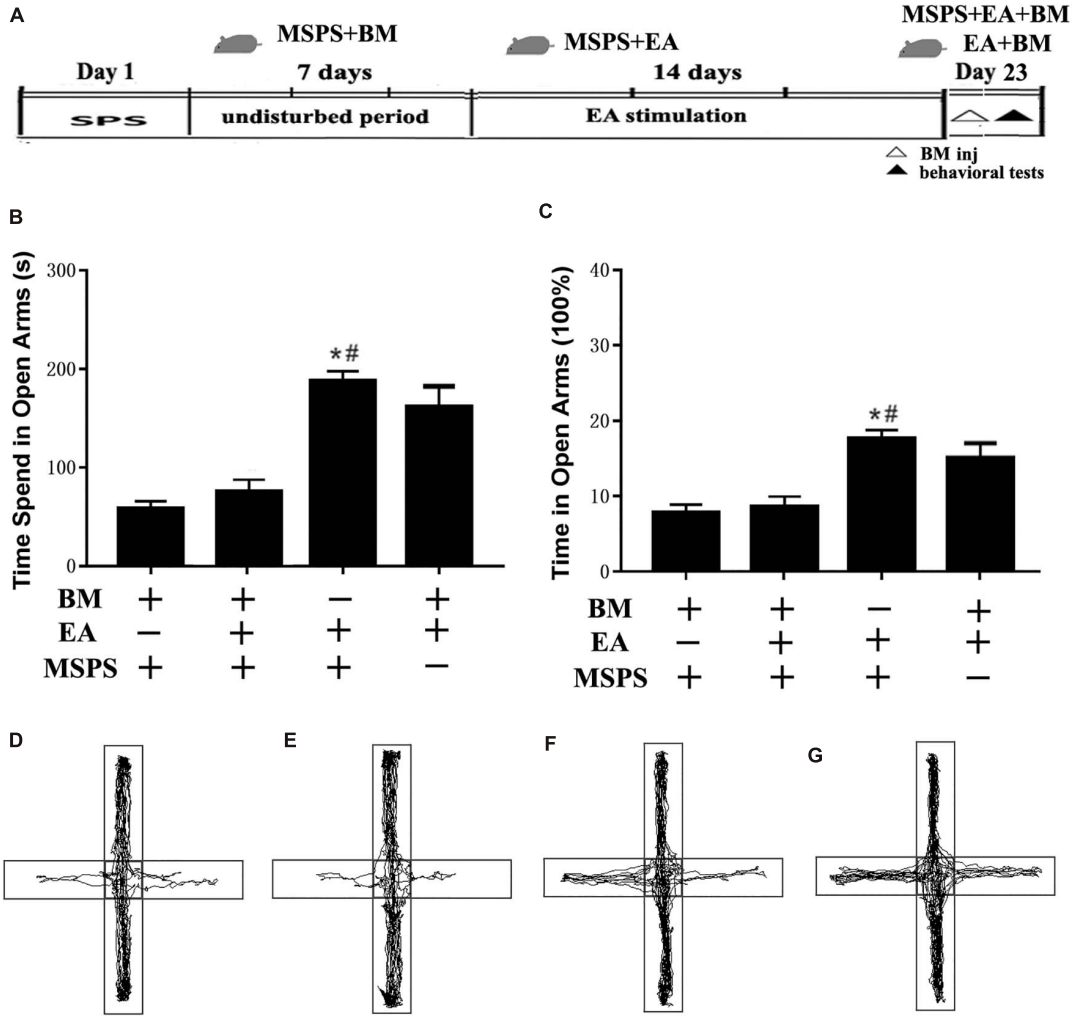

FIGURE 4 | Transient vmPFC inactivation disrupts the effects of EA on anxiety-like behaviors in MSPS model rats. **(A)** A timeline of MSPS modeling, EA treatment, stereotactic injection, and behavioral testing protocols. Briefly, MSPS modeling was performed on day 1. After a 7-day rest, rats were treated once per day as per an EA protocol (days 9–22). On day 23, rats were stereotaxically injected with artificial CSF (aCSF) or a 1.0 nmol/0.1 nmol mixture of baclofen and muscimol (BM) in the bilateral vmPFC immediately prior to behavioral testing. **(B)** Time spent in the open arms during elevated plus maze (EPM) testing for rats in the indicated groups. **(C)** Percentage of time spent in open arms. **(D–G)** Real-time movement traces during EPC testing: **(D)** BM + MSPS group; **(E)** BM + MSPS + EA group; **(F)** EA + MSPS group; **(G)** EA + BM group. *#$P < 0.05$ vs. the BM + MSPS group and the BM + MSPS + EA group.

(**Figures 6C,D**), with the number of spikes per burst in these four respective groups being 3.0 ± 0.43, 2.1 ± 0.25, 2.7 ± 0.25, and 2.4 ± 0.2. None of these variables differed significantly among these analyzed groups ($P > 0.05$).

Ventromedial prefrontal cortex stimulation was sufficient to evoke short- and long-latency excitatory and inhibitory responses in VTA DA neurons (**Figures 7A,B,F**). Of the 55 neurons analyzed in this study following vmPFC stimulation, 7 (12.7%) exhibited long-latency excitation, 24 (4.37%) exhibited short-latency excitation, 20 (36.3%) exhibited inhibition, and 4 (7.3%) exhibited no response. These results are in line with those of prior physiological studies assessing the impact of vmPFC stimulation on evoked DA neuron responses (Moorman and Aston-Jones, 2010). Chi-squared tests revealed no significant differences with respect to the types of VTA DA neuron responses in the four treatment groups ($P > 0.05$), nor were there any changes in mean firing rate over the course of vmPFC stimulation (**Figures 7C,D**). There were significant differences in bursting activity (%SIB) during ventral mPFC stimulation among treatment groups, with PTSD and SEA rats exhibiting no differences in %SIB between baseline and vmPFC stimulation (**Figures 7A,B**), whereas %SIB rose relative to baseline upon ventral mPFC stimulation in EA and control rats (*$P < 0.05$, **Figure 7E**).

DISCUSSION

Herein, we determined that EA treatment was sufficient to alleviate PTSD-associated anxiety-like behaviors in MSPS model rats. Immunofluorescent staining revealed that these anxiety-like

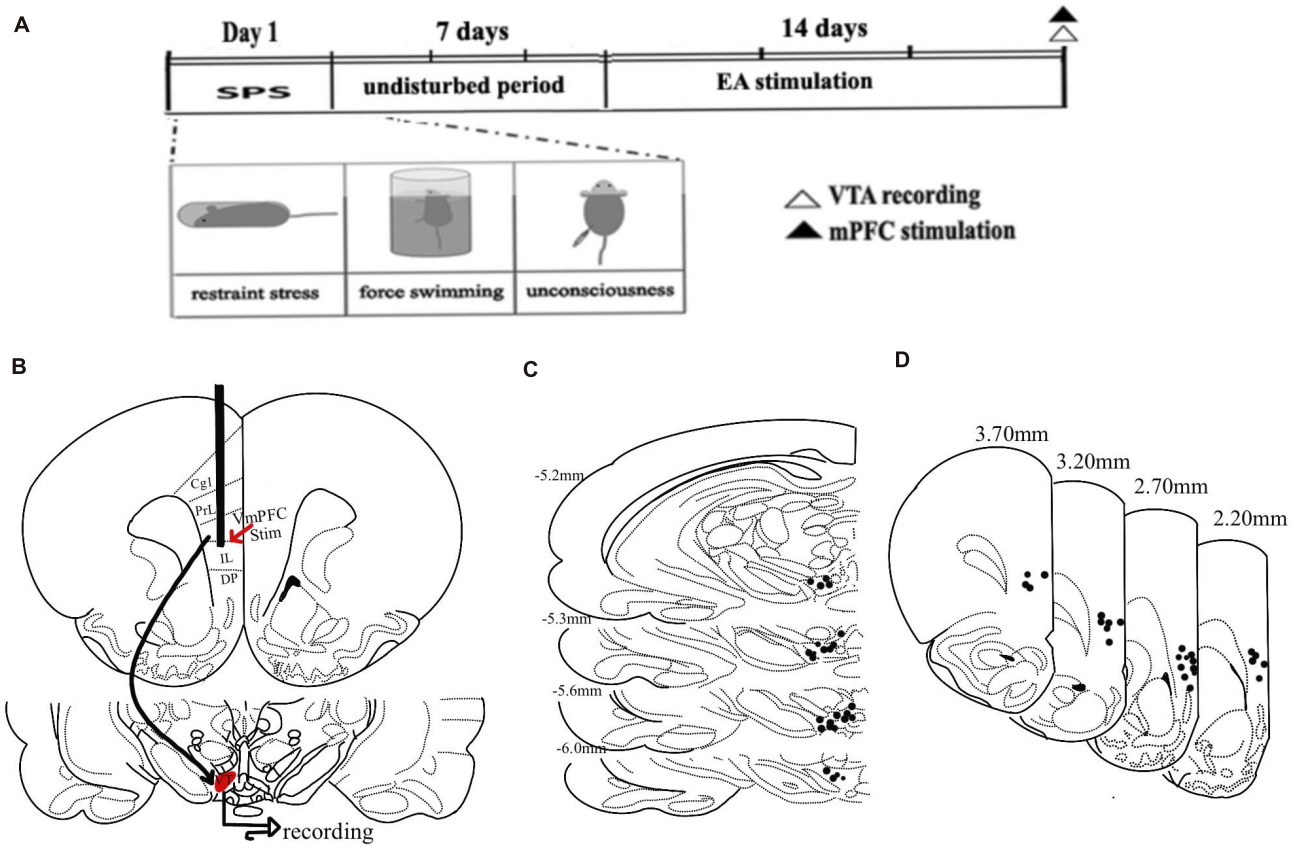

FIGURE 5 | Experimental timeline overview and vmPFC stimulation and recording site schematics. **(A)** A timeline of MSPS modeling, EA treatment, electrophysiological stimulation, and recording. **(B)** An overview of the vmPFC electrophysiological stimulation and recording sites used for this study. **(C)** Plots of 55 histologically localized DA neurons within the VTA. **(D)** Hemisection plots of the prefrontal cortex indicating stimulation site distributions.

behaviors were correlated with reduced vmPFC c-Fos expression, whereas EA treatment enhanced c-Fos levels in this region, consistent with functional vmPFC activation. Transient vmPFC inactivation ablated the ability of EA to alleviate anxiety-like behaviors. While PTSD model rats did not exhibit any change in VTA DA neuron %SIB upon vmPFC stimulation relative to baseline, such stimulation did increase the bursting activity of these neurons in rats in the EA treatment group. Together, our findings suggest that PTSD disrupts vmPFC activation and input into the VTA, potentially impacting the ability of the vmPFC to facilitate cognitive control of anxiety and thus giving rise to anxiety-like behaviors. EA-based therapy may thus be an effective treatment for PTSD owing to its ability to remediate pathological changes in the vmPFC and its inputs into the VTA.

The design of effective EA protocols is essential in order to effectively treat PTSD, with variables such as the choice of acupoints and the timing of treatment being of particular importance. Zhou et al. (2019) previously demonstrated the ability of EA pretreatment to prevent SPS-mediated induction of behaviors associated with anxiety. We and others have also previously shown that EA can similarly suppress anxiety-like behaviors following PTSD modeling (Liu et al., 2019). In this article, we found that a 14-day EA treatment period was sufficient to reduce the incidence of such behaviors, in line with prior results (Oh et al., 2018). These findings suggest that EA can benefit PTSD at a range of different stages during its development. Consistent with past reports (Zhou et al., 2019), we determined that low-frequency EA was able to suppress anxiety-like behaviors, suggesting that low-frequency stimulation may be more beneficial than high-frequency stimulation as a means of alleviating affective emotional and psychological states. Of course, this issue would need to be further clarified. According to traditional Chinese medicinal theory, the body contains over 300 acupoints, each of which is associated with its own therapeutic effects. The GV20, HT8, and HT7 acupoints are commonly selected when treating psychological or emotional disorders such as anxiety and depression (Oh et al., 2018; Zhou et al., 2019). In this study, we determined that EA conducted at the ST36 acupoints was sufficient to reduce the severity of anxiety-like behaviors in PTSD model rats. This acupoint is frequently used to treat pain, addiction, and disorders of the digestive system. These results suggest that this acupoint may also have value in the treatment of depression and anxiety. Additional study of synergistic combinations of ST36 and other acupoints may guide acupuncturists in the effective treatment of PTSD.

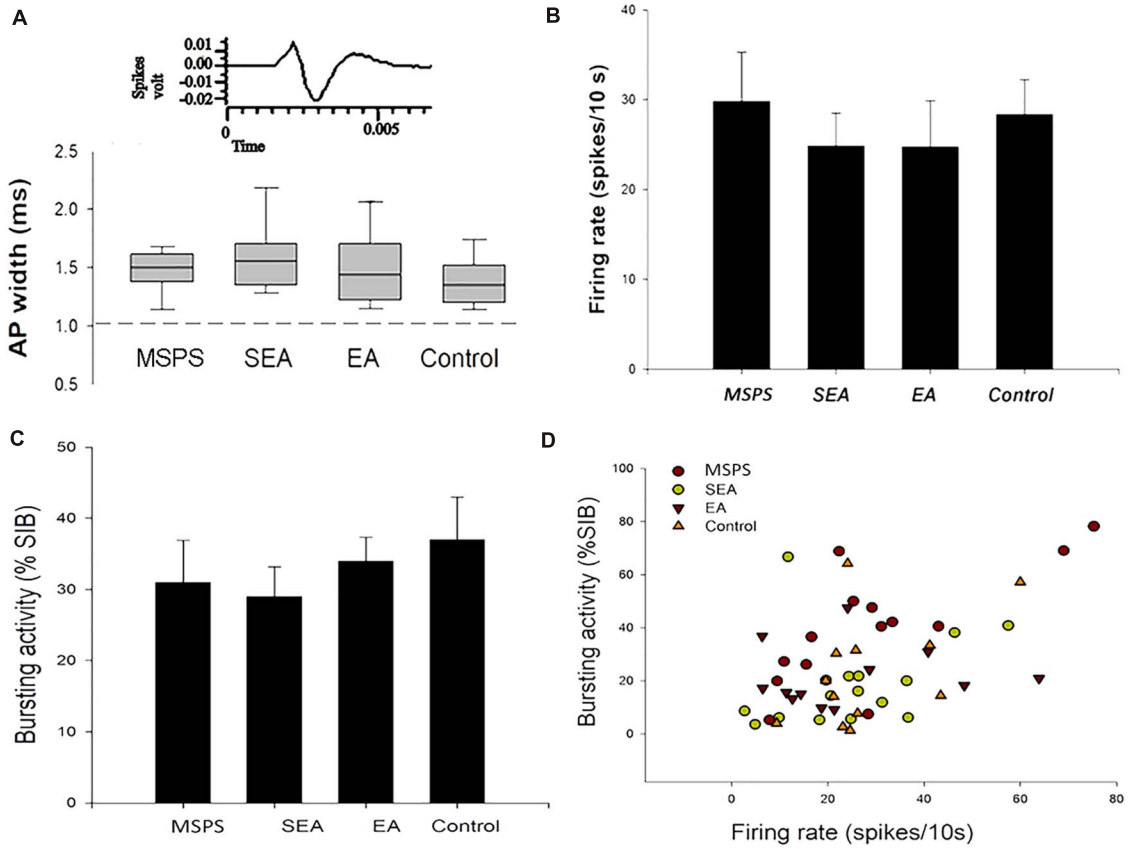

FIGURE 6 | MSPS and EA treatment alter VTA DA neuron responses. **(A)** Representative average extracellular neuron waveforms. The width of an action potential (AP) was measured from the start of that potential to the apex of the negative trough, and similar normal distributions of AP widths (range: 1.1–2.7 ms) were detected in PTSD rats, SEA rats, EA rats, and controls. **(B–D)** Average firing rates and bursting activity (%SIB) of VTA DA neurons in the indicated treatment groups.

Our results suggested that EA can improve anxiety-like behaviors in PTSD model animals through mechanisms associated with the vmPFC. The mPFC is generally separated into the dorsolateral (dlPFC) and ventromedial regions (Koenigs and Grafman, 2009), with the dlPFC receiving sensory cortex input and being densely interconnected with premotor areas, frontal eye fields, and the lateral parietal cortex (Barbas, 2000), whereas the vmPFC protections are primarily associated with the amygdala, hypothalamus, and periaqueductal gray matter. The dlPFC is associated with executive and cognitive functions, while the vmPFC has been linked to the top-down regulation of emotional processes, attention, and executive functions. PTSD patients exhibit structural and functional vmPFC abnormalities that may contribute to fear and anxiety responses (Yi et al., 2011; Calhoon and Tye, 2015). Indeed, individuals suffering from PTSD associated with interpersonal violence exhibited reduced vmPFC activity in response to emotional scenes in an fMRI study relative to controls. The observed reductions in Fos-positive neurons in the vmPFC of PTSD model rats in this study were in line with those observed in prior analyses (Yu et al., 2015; Pati et al., 2018). Behavioral analyses have suggested that the vmPFC contributes to anxiety-like behaviors, and acute pharmacogenomic vmPFC excitatory neuron activation can markedly decrease the incidence of these behaviors (Pati et al., 2018; Salvi et al., 2019). Similarly, vmPFC deep brain stimulation (DBS) can decrease anxiety-like behavior incidence in a model of PTSD (Reznikov et al., 2018). Acupuncture analgesia has recently been linked to cortical modulation (Oh et al., 2018). The anterior cingulate cortex, for example, is critically linked to the efficacy of EA in the treatment of formalin-induced inflammatory pain model rats (Yi et al., 2011). Our lab has previously demonstrated that acupuncture can influence vmPFC neuron firing activity (Zhang et al., 2017). Herein, we additionally determined that EA significantly enhanced vmPFC c-Fos expression in PTSD model rats, while transient vmPFC inactivation was sufficient to reverse the beneficial effects of EA on anxiety-like behaviors in these animals. These data suggest that EA may represent a viable treatment alternative to DBS as a means of activating the vmPFC to treat symptoms associated with PTSD.

Dopamine neuron activation within the VTA is crucial as a means of preventing generalized anxiety (Zweifel et al., 2011; DeGroot et al., 2020), highlighting the potential value of enhancing DA neurotransmission in order to treat PTSD. In a prior study, for example, SPS model mice were treated with DA D2/D3 receptor agonists and exhibited the attenuation of PTSD-like symptoms (Malikowska-Racia et al., 2019). DA neuronal

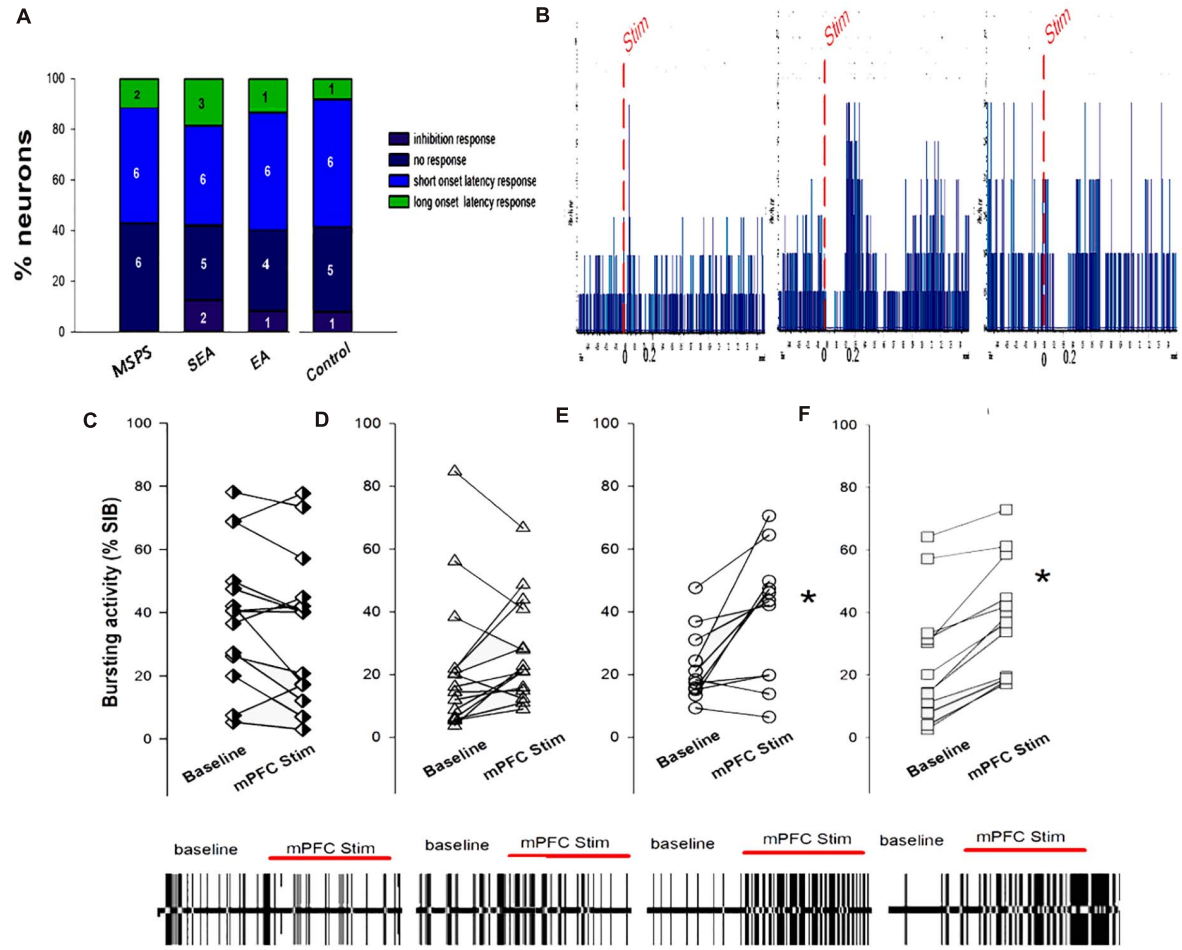

FIGURE 7 | The association between MSPS and EA treatment VTA DA neuron responses to vmPFC electrical stimulation. **(A)** VTA DA neuron response types in response to vmPFC stimulation in the indicated treatment groups. **(B)** Representative DA neuron short-latency excitatory responses (left), long-latency excitatory responses (middle), and inhibition responses (right) following vmPFC stimulation (50 pulses, 0.5 Hz). **(C–F)** VTA DA neuron bursting activity (%SIB) of VTA DA at baseline and upon vmPFC stimulation. **(C)** PTSD group; **(D)** SEA group; **(E)** EA group; **(F)** Control group. PTSD and SEA rats exhibiting no differences in %SIB between eight baseline and vmPFC stimulation, whereas %SIB rose relative to baseline upon vmPFC stimulation in EA and control rats. *$P < 0.05$, vs. baseline.

bursting results in significantly increased DA release, suggesting that this activity pattern is integral to the mesocorticolimbic DA system. Glutamatergic mPFC input to the VTA is also required for DA neuron burst activity (Murase et al., 1993; Tong et al., 1996), with this effect being dependent on NMDA receptor activation (Svensson, 2000). vmPFC stimulation activates VTA neurons (Gariano and Groves, 1988; Massi et al., 2008) and releases DA (Taber et al., 1995; You et al., 1998). Herein, we determined that PTSD model rats exhibited significantly decreased VTA DA neuron burst activity upon stimulation of the vmPFC, consistent with the disruption of mPFC inputs to the VTA as a consequence of this pathological condition. The anxiety-like behaviors associated with PTSD may thus be attributable to the abnormal functioning of this vmPFC-VTA neural circuit. A 14-day consecutive EA treatment regimen was sufficient to reverse these PTSD-associated reductions in VTA DA neuron burst activity, suggesting that the beneficial effects of EA on anxiety-like behaviors in this pathological context may be attributable to the impact of this therapeutic approach on VTA inputs from the vmPFC.

Certain factors must be considered when interpreting the results of our study. First, we utilized pentobarbital to anesthetize rats in our MSPS modeling approach, rather than ether, which is used for standard SPS modeling of anesthesia stress. We made this substitution owing to the fact that ether is both explosive and known to be toxic in humans and other mammals. In prior studies, no differences in gene expression, hematological findings, or biochemical parameters were observed when comparing treatments with ether, pentobarbital, and isoflurane (Nakatsu et al., 2017). Some researchers have reported that pentobarbital injection can induce additional stress that can interfere with cortisol levels in the plasma (Wu et al., 2015), and acute pentobarbital administration in rats is associated with the impairment of spatial learning, memory, and hippocampal long-term potentiation (Wang et al., 2015). In line with these reports, our PTSD model rats exhibited a range of anxiety-like

behaviors. In addition to the differences in OFT and EPM performance detailed above, these animals exhibited reductions in body weight and climbing frequency as well as elevated corticosterone levels relative to control rats (supplementary data). Second, studies of SPS model animals and humans with PTSD have reported reductions in vmPFC activity. We found that EA treatment was associated with increased expression of c-Fos within the vmPFC, while transient vmPFC inactivation in our PTSD model rats was sufficient to ablate the effects of EA treatment on anxiety-like behaviors. These findings suggest that the vmPFC is thus implicated in the impact of EA on PTSD-related anxiety. Neurons within the vmPFC are primarily classified as putative pyramidal neurons and interneurons (85 and 15%, respectively) (Homayoun and Moghaddam, 2007; Sotres-Bayon et al., 2012). SPS model rodents have been shown to exhibit reduced mPFC glutamate levels (Knox et al., 2010; Lim et al., 2017; Piggott et al., 2019), indicating that vmPFC glutamate levels may mediate the effects of EA on the anxiety-like behaviors of PTSD model animals, although further research will be necessary to confirm this possibility. Single-unit extracellular recordings from rodents have additionally provided critical insights into the computational roles of DA neuron firing (Schultz, 2016). It is important to note that these recordings were made while rats were under the effects of isoflurane-induced anesthesia, which is likely to impact baseline activity. However, it should not qualitatively impact the effects of pathway activation (Zimmerman and Grace, 2016). In addition, the stability of brain states under controlled anesthesia conditions represents an experimental advantage, given that it enabled the unbiased identification of DA neuron firing. Our results thus provide direct insight into candidate mechanisms that can be tested in conscious animals in future studies. The DA neurons in the present study were identified based upon basal firing rate and waveform shape. As our study was focused on a small subset of DA neurons, additional electrophysiological analyses are necessary to validate and expand on our findings. Even so, our data offer a novel and robust foundation for future research.

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 Shanghai University of Traditional Chinese Medicine Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

YH, XS, BY, and SL conceived the study design. YH, LL, MC, and CW contributed to perform research. YH, LL, HM, XQ, and SL helped to draft and revise the manuscript. YH, LL, MC and CW, and SL analyzed the data. All authors participated in writing the manuscript and all have read and approved the final manuscript.

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Personality Traits in Burning Mouth Syndrome Patients With and Without a History of Depression

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Objectives: So far, the strong link between neuroticism, chronic pain, and depression has been well-documented in literatures. Some suggested that they might share etiological factors, thus resulting in overlapping constructs. However, such effect has never been tested in burning mouth syndrome (BMS) patients, a complex phenomenon influenced by both neuropathic and psychopathological factors. We aim to clarify how personality affects individual's pain and pain-related experiences.

Methods: Two hundred forty-eight patients with BMS provided demographic information and psychiatric history; completed Ten-Item Personality Inventory, a Visual Analog Scale of pain, and McGill Pain Questionnaire; and provided adequate parameters of depressive state, catastrophizing thinking, and central sensitization.

Results: BMS patients with depression history suffered more severe clinical symptoms and scored higher in neuroticism and less in openness and extraversion than did those without psychiatric diagnoses. After age, sex, and duration of pain were controlled, neuroticism in BMS patients with depression correlates with affective dimension of pain. Instead, if psychiatric history is absent, neuroticism correlates with sensory dimension and pain intensity. In both groups, higher neuroticism, unlike other personality facets, contributed to a more severe clinical condition.

Conclusion: Of the five traits, neuroticism appears to be the most crucial dimension associated with the pain symptoms and patient's conditions. This study implies that management of pain must extend beyond solely providing pain-relieving medication and must require a holistic and multidisciplinary approach.

Keywords: big five traits, neuroticism, burning mouth syndrome, depression, catastrophizing, central sensitization

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INTRODUCTION

Burning mouth syndrome (BMS) is referred to as the most well-documented chronic oral pain. Its origin is largely multifactorial, with clinical symptoms ranging from simple burning pain of tongue to uncomfortable dysesthesia of other oral mucosal without evident signs (1, 2). Given the fact that BMS is a complex-to-handle, subjective experience, there lacks a gold standard in diagnosis and treatment. To date, neither pharmacotherapy nor psychotherapy could benefit all BMS patients. In a recent review, we discussed the efficacy of central neuromodulators in BMS

management and proposed the involvement of central sensitization, which is characterized by the hypersensitivity to stimuli, as a consequence of neural dysregulation and hyper-excitability in the central nervous system (2). This condition was theorized as the root pathophysiological mechanism of non-organic subgroup of functional somatic syndromes (e.g., fibromyalgia, irritable bowel syndrome, and nonspecific chronic low back pain), which may share common etiology with BMS (3).

In terms of personality, this multifaceted combination reflects individual differences in thoughts, behaviors, and ability to cope with life stressful events. Whereas, previous studies suggest a relation between specific pain-prone personality characterized by a high level of neuroticism [one of the five factors in the five-factors model (FFM)] and BMS, its effects on pain perception are still controversial (4–7). There also remains a question of whether the relationship between personality and pain experiences in BMS is causality or merely correlation (8). While Grushka et al. suggested that personality disturbances tend to increase with a higher level of pain (7), other researchers theorize that certain personality traits predispose the development of chronic pain syndromes and contribute to how a person perceives and appraises pain (9, 10). In particular, a high neurotic person has a lower pain threshold and higher tendency to catastrophize (11). Notably, existing research on BMS tends to signify the role of psychopathological aspects rather than focus on personality and cognitive-affective constructs in this population (4).

Among psychiatric comorbidities in BMS, depression is the most common, with reported prevalence from 27.2 to 56.7% (12, 13). So far, the strong-linked relationship between neuroticism, pain, and chronic depression has been well-documented. Some suggested that they might share etiological factors, thus resulting in overlapping constructs (14, 15). Other studies argue that depressive symptoms might increase neuroticism and decrease extraversion, mainly at small and limited state effects (16, 17). Indeed, neuroticism is said to have biological origin, to mature until early adulthood, and then remain relatively consistent over time (especially in comparison with other psychological distress like depression or anxiety) (18–20). Altogether, it raises the question of how personality, pain, and depression interact. Current literature does not show us a clear picture, and yet such kind of effect has never been tested in BMS patients, particularly between patients with and without depression comorbidity. The analysis of this complex intertwining framework is therefore critical to the implication of treatment, by identifying at-risk individuals and tailoring approaches.

Hence, our objectives were as follows: (1) compare pain characteristics, depressive state, pain-related catastrophizing, central sensitization, and personality features among BMS patients with and without a history of depression; (2) determine the extent to which personality traits influence pain (intensity, component of sensory, or affective), depressive state, pain-related catastrophizing, and central sensitization.

METHODS

Procedures and Participants

Participants of the study were recruited among patients who visited the Psychosomatic Dentistry Clinic at Tokyo Medical and Dental University in Tokyo, Japan, for the first time between July 2018 and April 2020. Inclusion criteria were as follows: (1) age over 18; (2) had no difficulty communicating in Japanese; and (3) had a diagnosis of BMS. The diagnosis was based on the definition of "a chronic intraoral burning sensation that has no identifiable cause either local or systemic condition or disease," proposed by the International Association for the Study of Pain (21). The unexplained nature of burning symptoms was confirmed by a comprehensive step-by-step examination and necessary laboratory tests. Patients were excluded if they had conditions that hinder reliable data collection (e.g., neurodevelopmental disorder, dementia, and difficulty in reading, understanding, and/or answering questions). Informed written consent was obtained from all the patients, and the study protocol was approved by the Ethical Committee of Tokyo Medical and Dental University (D2013-005).

Parameters

The following sociodemographic information was collected: age, sex, and duration of illness. Psychiatric history was obtained from referral letters from the patients' psychiatrists. At the first visit, we required all patients to submit referrals if they had experienced any history of psychiatric disorder. The diagnosis was then adopted as given by their attending psychiatrists. In case the patients reported such history but could not provide referrals (for any reason), we then directly contacted their psychiatrists to inquire further details. Patients with different psychiatric diagnoses other than depression (depressive disorders, *Diagnostic and Statistical Manual of Mental Disorders*, 5th ed.) were then excluded, leaving the final two groups of BMS patients: one with a history of depression and the other without any history of psychiatric disorder.

In this present study, we assessed the personality of BMS patients using the FFM (22). This framework comprises extraversion, agreeableness, conscientiousness, neuroticism, and openness. FFM is one of the most used and studied models of personality. All patients answered the Japanese version of the Ten-Item Personality Inventory (TIPI-J), a concise validated questionnaire of FFM. Each of the 10 items was rated from 1 (strongly disagree) to 7 (strongly agree) (23).

The pain characteristics were examined using the short form of the McGill Pain Questionnaire (SF-MPQ) (24). It contains 15 descriptors: 1 to 11 represent the sensory dimension, and 12 to 15 represent the affective dimension. The Visual Analog Scale (VAS) indicating pain severity was 10 cm in length and anchored by 0 (no burning pain) and 100 (worst pain imaginable).

Patients also completed a 20-item assessment tool known as Zung's Self-Rating Depression Scale (SDS) for the current depressive state (25). Responses range from 1 ("a little of the time") to 4 ("most of the time"), and the total score ranges between 20 and 80.

Patients' feelings and attitudes toward the painful situation (i.e., the tendency toward rumination, magnification, and helplessness due to BMS) were examined using the Pain Catastrophizing Scale (PCS). Patients answered 13 items in total, each of which is rated on a 5-point Likert scale (from 0 "not at all" to 4 "all the time") (26). In total, score ranges from 0 to 52.

To assess central sensitization severity levels in patients with BMS, we used a high reliability and validity screening instrument named Central Sensitization Inventory (CSI) (27). It consists of 25 questions on overlapping health-related symptom dimension of central sensitization, and patients are to answer using a scale from 0 (never) to 4 (always). A cumulative score ranges between 0 and 100.

Statistical Analysis

The statistical analysis was performed using R version 3.4.2 for Mac OS (R Foundation for Statistical Computing, Vienna, Austria). Probability values of $p < 0.05$ were accepted as statistically significant. Missing values were imputed ("MICE" package, R Foundation for Statistical Computing) in accordance with the precondition that they comprise <5% of total dataset and are random. We applied the chi-square test and the Kruskal–Wallis test to compare clinical characteristics (pain characteristics, depressive state, and pain-related catastrophizing), central sensitization severity level, and personality between the two BMS patient groups. A correlation matrix of Kendall's rank coefficients τ showing pairwise associations between personality and clinical characteristics was built. All the p-values were then adjusted for multiple tests, using the Holm–Bonferroni method (28). Afterward, we performed multivariate linear regression models to measure the degree of the associations between each personality dimension and a series of clinical characteristics (controlling for covariates: age, sex, duration of illness). In each model, one of five TIPI traits is the independent factor with age, sex, and illness duration, while six clinical parameters are the dependent variables. This model entails an additional variance–covariance matrix of the model coefficients. This helps estimate whether an independent variable jointly contributed to multiple dependent variables and also represents multicollinearity if the variables are highly correlated. Subsequently, the regression validation is conducted visually via regression diagnostic graphs, meaning evaluating the regression assumptions (e.g., the residuals are normally distributed, and their variance does not change as a function of X).

RESULTS

Baseline Characteristics

Data of 343 patients were reviewed. Ten patients were excluded due to conditions that could affect the reliability of their self-reports (schizophrenia, 4; dementia, 1; Alzheimer's disease, 2; mild cognitive impairment, 1; intellectual disability, 1; and Parkinson's disease, 1). Eighty-five with history of psychiatric disorders other than depression were omitted (anxiety disorders, 19; insomnia disorders, 18; somatic symptom and related disorders, 15; bipolar and related disorders, 3; obsessive-compulsive disorders, 1; unknown, 38). After the application of all criteria, data for 248 patients were available for analysis. The patients' mean age was 62.07 ± 13.53 years, and 81.5% of them were female. Patients had experienced pain for average 35.56 ± 46.63 months. The demographic characteristics, comorbid psychiatric disorders, and symptom-related parameters of 248 patients with BMS are shown in **Table 1**. Of 248 patients, 55 (22.2%) have experienced visiting a psychiatrist and were diagnosed as having major depressive disorder (90.9%) or dysthymia (9.1%).

TABLE 1 | Demographic characteristics and symptom-related parameters of 248 BMS patients.

	***N* (%) or Mean ± SD**
Female	202 (81.5)
Age (year)	62.07 ± 13.53
Duration of symptom (month)	35.56 ± 46.63
No psychiatric history	193 (77.8)
Depressive disorders	55 (22.2)
Major depression disorder	50 (90.9)
Dysthymia	5 (9.1)
SF-McGill Pain Characteristics	
Sensory dimension	2.58 ± 2.98
Affective dimension	4.62 ± 4.9
Pain intensity (VAS)	48.34 ± 27.7
Depressive state (SDS)	42.56 ± 10.7
Catastrophizing thinking (PCS)	28.33 ± 11.88
Central sensitization (CSI)	24.36 ± 14.22

SD, Standard Deviation; VAS, Visual Analog Scale; SDS, Self-Depression Scale; PCS, Pain-related Catastrophizing Scale; CSI, Central Sensitization Inventory.

Comparison of Clinical Characteristics, Central Sensitization Level, and Personality Traits by the Presence of Depression History

Despite no significant differences in demographic data (age, sex, and duration of illness: all p-values > 0.05), BMS patients with history of depression score higher in every aspect of clinical characteristics (pain, depressive state, and catastrophizing) and central sensitization level than those without psychiatric history (**Figure 1A**). Regarding the big five traits, the BMS group with history of depression had a significantly higher score in neuroticism ($p < 0.001$), but a lower score in openness and extraversion ($p < 0.05$). Other traits had differences, but no statistical significance was detected (**Figure 1B**). As seen in **Table 2**, there are different significantly positive correlations between scores of VAS, sensory, affective component of SF-MPQ, and SDS, PCS, and CSI in each group. However, all τ coefficients were higher in the group of patients with depression comorbidity.

The Associations Between Personality Traits and Pain, Depressive State, Pain-Related Catastrophizing, and Central Sensitization Level

In group of BMS patients without psychiatric history, after sex, age, and duration of illness were controlled, significant associations were found between the following: neuroticism and sensory component of SF-MPQ ($\beta = 0.24$, $p < 0.01$, adj $R^2 = 0.053$, RMSE = 2.53), VAS ($\beta = 1.70$, $p < 0.05$, adj $R^2 = 0.04$, RMSE = 26.71), SDS ($\beta = 1.75$, $p < 0.001$, adj $R^2 = 0.164$,

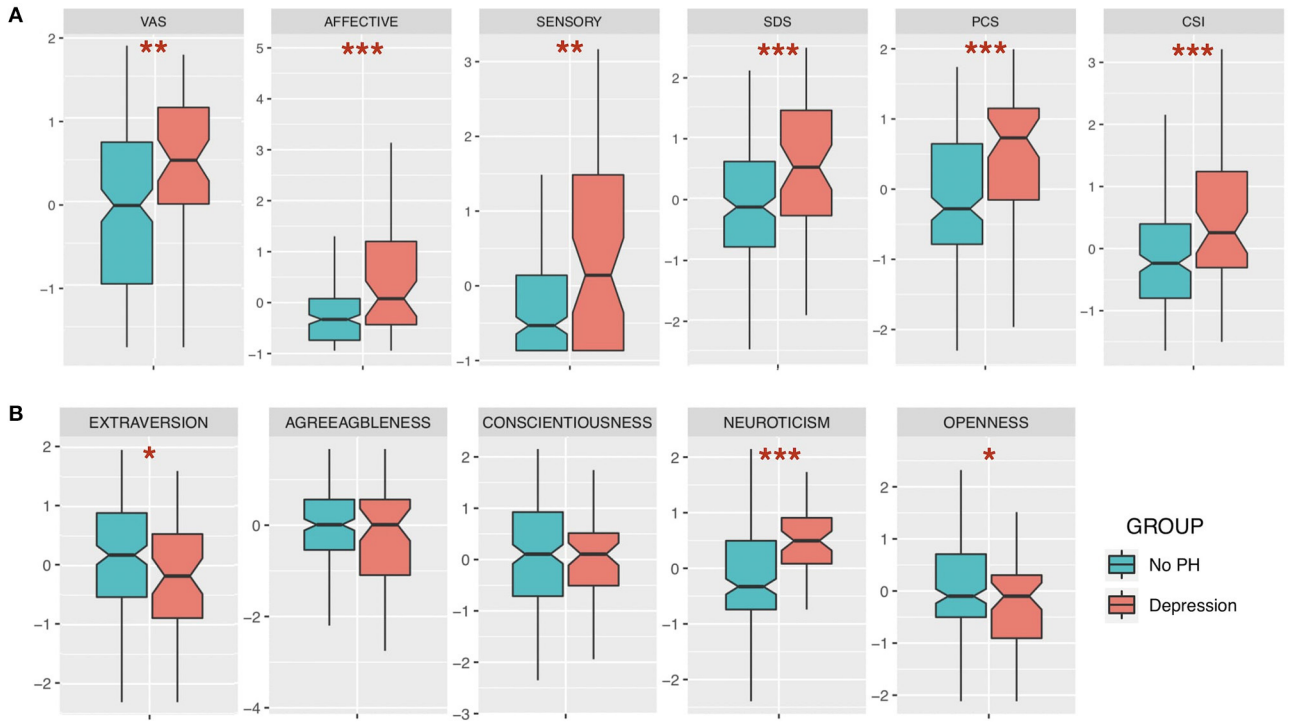

FIGURE 1 | Comparisons between groups of burning mouth syndrome (BMS) patients with depression history and without psychiatric history (standardized). **(A)** Clinical measures of pain, pain characteristics, depressive state, catastrophizing, and central sensitization. **(B)** Big five traits. VAS, Visual Analog Scale; SDS, Self-Rating Depression Scale; PCS, Pain-related Catastrophizing Scale; CSI, Central Sensitization Inventory. Wilcoxon rank sum test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

RMSE = 8.88), PCS ($\beta = 1.71$, $p < 0.001$, adj $R^2 = 0.1224$, RMSE = 10.77), and CSI ($\beta = 2.09$, $p < 0.001$, adj $R^2 = 0.153$, RMSE = 11.95). In contrast, a higher level of agreeableness was negatively associated with SDS ($\beta = -1.94$, $p < 0.001$, adj $R^2 = 0.095$, RMSE = 9.24) and CSI ($\beta = -2.16$, $p < 0.001$, adj $R^2 = 0.087$, RMSE = 12.40). Interestingly, SDS is the only psychological parameter that was associated significantly with all five traits (extraversion, $\beta = -0.57$, $p < 0.05$, adj $R^2 = 0.016$, RMSE = 9.63; conscientiousness, $\beta = -0.80$, $p < 0.01$, adj $R^2 = 0.029$, RMSE = 9.57; openness, $\beta = -0.90$, $p < 0.01$, adj $R^2 = 0.040$, RMSE = 9.52). None of the associations between pain characteristics and traits other than neuroticism were statistically significant. For details, see **Table 3**.

Moreover, if a history of depression is present, after covariates were adjusted, a higher score in neuroticism significantly correlates with a higher level of affective component ($\beta = 0.85$, $p < 0.05$, adj $R^2 = 0.050$, RMSE = 5.44), whereas it has no relation with either sensory component of SF-MPQ or VAS (**Table 3**). Also, SDS and extraversion are no longer significantly correlated. So are agreeableness and conscientiousness.

If we define a higher level of VAS, SDS, PCS, and CSI as a negative impact (i.e., patients suffer from more severe pain, depressive state, and negative thinking and have higher central sensitization level), the results in parts A and B in **Table 3** consistently show the opposite effects by neuroticism vs. the group of four other traits. Higher neuroticism contributes to worse patient conditions. Higher extraversion, agreeableness, conscientiousness, and openness are associated with less severe conditions, but their impacts vary.

DISCUSSION

Among many types of chronic pain, those without obvious pathology like BMS are the most difficult to deal with, thus inducing recurring expenses. As long as the dilemma of the origin BMS remains unsolved, the key to any successful treatment is still a puzzle. So far, this is the largest FFM-based personality observation in one population of BMS, in which we seek to address how individual traits associate with patients' clinical experiences and central sensitization level. Our main findings include (1) patients with a history of depression suffering from more severe clinical symptoms, had higher central sensitization, and scored higher in neuroticism and lower in extraversion and openness than those without psychiatric diagnosis; (2) neuroticism and the other four traits have inverse correlations with pain, depressive state, catastrophizing thinking, and central sensitization; and (3) higher neuroticism combined with a history of depression results in a higher level of affective dimension, while a higher level of sensory dimension and pain intensity is observed in those without psychiatric history.

Since depression and pain share biological pathways, their overlapping presence is commonly observed. Findings from

TABLE 2 | Correlations between measures (unstandardized, Kendall's rank correlation *tau*, *P-values* were adjusted for multiple tests).

	E	A	C	N	O	VAS	Affective	Sensory	SDS	PCS	CSI
(A) BMS patients without psychiatric history											
1. Extraversion		0.13	0.13	−0.16	0.19	0.00	−0.01	−0.04	−0.10	−0.05	−0.06
2. Agreeableness			0.28**	−0.21	−0.01	0.04	0.06	0.05	−0.21	0.02	−0.16
3. Conscientiousness				−0.22	0.09	0.01	0.07	0.07	−0.14	−0.02	−0.05
4. Neuroticism					−0.16	0.11	0.09	0.09	0.31***	0.22	0.26*
5. Openness						−0.01	0.08	−0.01	−0.15	−0.07	0.01
6. VAS							0.40***	0.35***	0.14	0.24*	0.15
7. Affective								0.43***	0.14	0.28**	0.23
8. Sensory									0.22	0.35***	0.22
9. SDS										0.29***	0.34***
10. PCS											0.31***
11. CSI											
(B) BMS patients with a history of depression											
1. Extraversion		0.03	0.21	−0.12	0.27	−0.15	−0.11	−0.14	−0.20	−0.20	−0.24
2. Agreeableness			0.34	−0.21	−0.16	0.02	−0.13	−0.12	−0.10	−0.11	−0.24
3. Conscientiousness				−0.25	−0.09	−0.08	−0.13	−0.15	−0.11	−0.23	−0.22
4. Neuroticism					−0.03	0.05	0.29	0.08	0.20	0.30	0.19
5. Openness						−0.15	−0.03	−0.03	−0.20	−0.03	0.03
6. VAS							0.47*	0.52**	0.25	0.39	0.26
7. Affective								0.54**	0.21	0.40	0.39
8. Sensory									0.22	0.47*	0.33
9. SDS										0.42	0.49**
10. PCS											0.46*
11. CSI											

−1 −0.4 −0.2 −0.1 0 0.1 0.2 0.4 1.

VAS, Visual Analog Scale; SDS, Self-Depression Scale; PCS, Pain-related Catastrophizing Scale; CSI, Central Sensitization Inventory.
**P < 0.05; **P < 0.01; ***P < 0.001 (adjusted with Holm-Bonferroni method).*

TABLE 3 | Impacts of personality on clinical measures (controlled for age, sex and illness duration).

	Extraversion	Agreeableness	Conscientiousness	Neuroticism	Openness
BMS patients without psychiatric history					
SF-McGill pain characteristics					
Sensory dimension	−0.08	−0.19	0.05	0.24**	0.03
Affective dimension	0.02	−0.08	0.12	0.15	0.25
Pain Intensity (VAS)	−0.16	0.08	0.04	1.70*	0.03
Depressive state (SDS)	−0.57*	−1.94***	−0.80**	1.75***	−0.90**
Catastrophizing thinking (PCS)	−0.23	−0.64	−0.29	1.71***	−0.28
Central sensitization (CSI)	−0.61	−2.16***	−0.13	2.09***	0.11
BMS patients with a history of depression					
SF-McGill pain characteristics					
Sensory dimension	−0.25	−0.38	−0.26	0.16	−0.13
Affective dimension	−0.30	−0.24	−0.18	0.85*	−0.15
Pain intensity (VAS)	−2.07	0.17	−1.50	1.80	−2.41
Depressive state (SDS)	−1.12	−1.26	−1.07	1.50*	−1.46*
Catastrophizing thinking (PCS)	−0.89	−0.46	−1.07	1.50*	−0.31
Central sensitization (CSI)	−1.79*	−2.66*	−1.67	1.60	0.27

VAS, Visual Analog Scale; SDS, Self-Depression Scale; PCS, Pain-related Catastrophizing Scale; CSI, Central Sensitization Inventory.
*Multivariate linear regression; *P < 0.05; **P < 0.01; ***P < 0.001.*
All regression models were validated visually using regression diagnostic graphs.

chronic pain cohort studies assessing the impact of depression are in agreement with ours. This demonstrates a comorbidity of depression significantly exacerbating pain complaint, especially pain intensity, and greater impairment (29). A part of our finding is also consistent with previous work by Kim et al., suggesting that BMS patients with psychological problems experienced more intensive pain, higher stress-related symptoms, and more difficulties in daily life (30). However, in most of the cases, the questions often focus on estimating depression's prevalence and its impact on healthcare cost, rather than comparing pain-related experiences (31).

Despite unknown and ambiguous biological mechanism, personality is considered an independent efficient marker to predict overall health and well-being (14, 32, 33). Personality dysfunction has been also reported as "the most clinically salient problem" in patients with medically unexplained symptoms such as uncertain etiology like BMS (34). In terms of neuroticism, this trait is also referred to as emotional instability—a normal personality dimension that varies between individuals. It represents the degree to which a person interprets an ordinary life circumstance as threatening, negative, and unsafe. According to Smith et al., a person with high neuroticism profile has a lower threshold when perceiving a pain stimulus as threatening; thus, pain-related catastrophizing emerges (32). In agreement with our findings, Goubert et al. found that neuroticism significantly correlates with catastrophizing, vigilance of pain, and fear of movement (11). Moreover, due to evidence showing the influence of neuroticism on pain perception, some authors hypothesized the involvement via central sensitization. According to Woolf, there remains a question of "whether there are individuals with a higher inherited propensity for developing central sensitization than others" (35). A certain profile of personality might be the potential answer for those individual differences.

Of particular interest of this study is that in BMS patients with depression and high neuroticism score profiles, pain seems to be perceived as a more affective disturbance. In contrast, patients without psychiatric disorders have the sensory component elevated by higher neuroticism. A similar result was observed by Harkin et al., in which highly neurotic patients perceived their chronic pain as more disturbing than less neurotic group did, despite no difference in the sensation magnitude. However, no impact of psychiatric comorbidity was examined (36). Our findings could also be explained by prior fMRI study in fibromyalgia patients, illustrating that the presence of depression significantly associated with enhanced neural activities in the amygdalae and contralateral anterior insula, where the affective dimension is processed (37). Nevertheless, to understand thoroughly the "transition" impact of neuroticism on pain perception from sensory dimension to an affective one in BMS patients with depression comorbidity, a more comprehensive prospective design is necessary.

Although there exists debate on whether the effect of neuroticism is overreported, evidences supporting its negative role in chronic conditions have been more consistent than those of four other traits (11, 38–40). In our study, the other traits showed no or little impact on pain parameters; instead, they were partially correlated with depressive state, pain catastrophizing, and central sensitization through a variety of levels, but much less than neuroticism. Altogether, we suggest that the influence of personality on symptoms of BMS seems to be mainly driven by neuroticism.

Strength and Limitation

This current study was limited in three main facets. The retrospective design without a control group is unable to define whether neuroticism affects chronic pain patients differently. Another potential bias is that the data were collected from a specific clinic of academic dental hospital where patients are likely to have more complex, multiple chronic conditions and suffer from more severe burning pain. Such sample might not represent the general population of BMS. Moreover, factors related to the history of depression (such as clinical definition, treatment, and response) were not fully addressed in the current study, which limit the result interpretation. The final concern is with regard to the low R^2 in multivariate regression models. However, this might be expected, since a combination of one personality trait, age, gender, and duration might not predict very well the variation of clinical outcomes. Nevertheless, it should be noted that our study is the first one to assess the relationship of personality, pain, and depression, with the largest sample of patients among personality-related research on BMS so far.

CONCLUSION

BMS patients with history of depression appear to have personality profiles different from those of the group without psychiatric history. Of the five traits, neuroticism appears to be the most crucial dimension associated with clinical experiences in BMS patients. Notably, patients with depression comorbidity and a high neuroticism score profile seem to perceive pain as affective disturbance. This study reinforces that management of pain must extend beyond solely providing pain-relieving medication and require a holistic and multidiscipline approach. Further study needs to address the role of personality in predicting patients' adherence and treatment outcome in a long-term follow-up.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available due to ethical concerns and security requirements of patients-related data. Requests to access the datasets should be directed to Dr. Trang Thi Huyen Tu, tu.ompm@tmd.ac.jp.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of Tokyo Medical and Dental University. The patients/participants provided their written informed consent to participate in this study. The study protocol was approved by the Ethical Committee of Tokyo Medical and Dental University (D2013-005) and conformed to the provisions

of the Declaration of Helsinki. All patients had been informed about the possibility of their data being used for study purposes at their first visit and had provided written informed consent.

AUTHOR CONTRIBUTIONS

TT, HM, and AT were involved in study design, data collection, data analysis, and manuscript drafting. MW, TS, CH, CT, and MT contributed in data collection, results interpretation, and manuscript revision. All authors read and approved the manuscript.

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TNF-Alpha as an Initiator of Allodynia and Anxiety-Like Behaviors in a Preclinical Model of PTSD and Comorbid Pain

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Post-Traumatic Stress Disorder (PTSD) is a debilitating mental health disorder that occurs after exposure to a traumatic event. Patients with comorbid chronic pain experience affective distress, worse quality of life, and poorer responses to treatments for pain or PTSD than those with either condition alone. FDA-approved PTSD treatments are often ineffective analgesics, requiring additional drugs to treat co-morbid symptoms. Therefore, development of new treatment strategies necessitate a better understanding of the pathophysiology of PTSD and comorbid pain. The single prolonged stress (SPS) model of PTSD induces the development of persistent mechanical allodynia and thermal hyperalgesia. Increased Nociceptin/Orphanin FQ (N/OFQ) levels in serum and CSF accompany these exaggerated nociceptive responses, as well as increased serum levels of the pro-inflammatory cytokine tumor necrosis factor (TNF-α). Therefore, the primary goal was to determine the role of TNF-α in the development of SPS-induced allodynia/hyperalgesia and elevated serum and CNS N/OFQ using two approaches: TNF-α synthesis inhibition, and blockade with anti-TNF-α antibody that acts primarily in the periphery. Administration of TNF-α synthesis blocker, thalidomide (THL), immediately after SPS prevented increased TNF-α and development of allodynia and hyperalgesia. The THL effect lasted at least 21 days, well after thalidomide treatment ended (day 5). THL also prevented SPS-induced increases in serum N/OFQ and reversed regional N/OFQ mRNA expression changes in the CNS. Serum TNF-α increases detected at 4 and 24 h post SPS were not accompanied by blood brain barrier disruption. A single injection of anti-TNF-α antibody to male and female rats during the SPS procedure prevented the development of allodynia, hyperalgesia, and elevated serum N/OFQ, and reduced SPS-induced anxiety-like behaviors in males. Anti-TNFα treatment also blocked development of SPS-induced allodynia in females, and blocked increased hypothalamic N/OFQ in

males and females. This suggests that a peripheral TNF-α surge is necessary for the initiation of allodynia associated with SPS, as well as the altered central and peripheral N/OFQ that maintains nociceptive sensitivity. Therefore, early alleviation of TNF-α provides new therapeutic options for investigation as future PTSD and co-morbid pain treatments.

Keywords: hyperalgesia, allodynia, nociceptin/orphanin FQ (N/OFQ), traumatic stress, thalidomide, NOP receptor, anti-TNF alpha antibody

INTRODUCTION

Chronic or persistent pain is one of the most commonly co-occurring physical problems for patients with PTSD, and an even higher incidence of this comorbidity was observed in the veteran population (1). Further complicating this co-morbidity are findings that patients with chronic pain and PTSD experience more intense pain and affective distress, higher levels of life interference, and greater disability than patients with either condition alone (2–4). Unfortunately, the trigger for development of PTSD and co-morbid pain symptoms is unknown, but findings by us and others using the single prolonged stress (SPS) model of PTSD suggest that tumor necrosis factor-α (TNF-α) initiates the development of pain and anxiety-like behaviors. Critically, this has not been tested directly. Nociceptin/Orphanin FQ (N/OFQ) is an opioid peptide that binds to fourth member of the opioid receptor superfamily, the N/OFQ peptide (NOP) receptor (5). N/OFQ and the NOP receptor are located in brain regions and nerve endings mediating pain sensitivity (6). Peripheral release of N/OFQ increases macrophage and monocyte infiltration, contributing to inflammation and nociceptive hypersensitivity (7). Therefore, because N/OFQ can bi-functionally modulate pain sensitivity, it is important to understand how N/OFQ is modulated under conditions that increase pain sensitivity and allodynia.

The single prolonged stress (SPS) model of PTSD has been employed as a preclinical model of PTSD for over 20 years (8, 9). Previous work in our lab and that of others has shown that SPS induces the development of persistent mechanical allodynia and thermal hyperalgesia (10), visceral hypersensitivity (11) and stress- (12) and surgically-induced hypersensitivity (13, 14). Appearance of increased nociceptive sensitivity in this model paralleled increased TNF-α acutely in serum (14–16). Subsequent to development of allodynia and hyperalgesia, N/OFQ levels increased in serum, CSF, PAG, hippocampus, and hypothalamus (10, 17, 18). Our lab demonstrated that mechanical allodynia and thermal hyperalgesia induced by SPS lasts at least 30 days in male and female rats, and is blocked by NOP receptor antagonists or absence of the NOP receptor (10, 17, 18). Interestingly, TNF-α increases N/OFQ expression (19, 20). Elevated circulating TNF-α has been noted in PTSD patients (including non-combat related trauma patients) (21, 22). TNF-α production in peripheral blood mononuclear cells (PBMCs) from PTSD patients was increased in response to LPS (23), compared to cells from non-PTSD control subjects, suggesting sensitization of the immune response with PTSD. The caveat of this comparison is that clinical data may be collected months-years after the initial traumatic event, so the timing of the increase in TNF-α differs from experimentally derived data. Further, the frequency and severity of PTSD symptoms correlates with circulating levels of several different cytokines besides TNF-α, including IL-1β (21, 22).

Anti-TNF-α therapy in rheumatoid arthritis patients decreases brain-derived TNF-α and alleviates pain (24). Consequently, drugs that reduce TNF-α synthesis may reduce pain, anxiety and depressive symptoms by moderating TNF-α-induced changes in neurotransmission (25). Reduced hyperalgesia and/or anxiety behaviors following SPS correlated with reduced circulating and hippocampal TNF-α and other inflammatory cytokines 1–2 weeks post-SPS (13, 26). However, those studies did not directly target TNF-α synthesis or activity. The primary goal of this study was to test the hypothesis that blockade of TNF-α synthesis or action shortly after initiation of a traumatic stressor (SPS) would prevent development of mechanical (tactile) allodynia, thermal hyperalgesia and subsequent elevation of N/OFQ. A secondary goal of the study was to evaluate anxiety-like behaviors.

MATERIALS AND METHODS

Animals

Adult Sprague–Dawley rats weighing 220–250 g at the initiation of SPS were obtained from Charles River Labs (Wilmington, MA). Animals were housed in the animal facility under a 12-h light: 12-h dark cycle (lights on at 06:00 h) with free access to food and water. After arrival, rats were acclimated to the animal facility for 7–10 days prior to initiation of experiments. Experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center and the US Army Medical Research and Materiel Command Animal Care and Use Review Office. Research was compliant with the Animal Welfare Act Regulations and other Federal Statutes relating to animals and experiments involving animals, and adhered to the principles set forth in the Guide for Care and Use of Laboratory Animals, National Research Council, 1996. All experiments conformed to the guidelines of the International Association for the Study of Pain. Every effort was made to minimize animal discomfort and reduce the number of animals used.

SPS

Animals were randomized into groups. The SPS procedure was followed as described (8, 27) with modification (10). After acclimatization, rats were exposed to complete restraint in

disposable plastic holders for 2 h, followed by grouped (3–4 rats) forced swimming for 20 min in a cylindrical plexiglass tank (46 cm tall × 20 cm in diameter) filled with 22°C water to a depth of 30 cm. After a 15 min recovery and drying period, animals were exposed to diethyl ether in a fume hood until consciousness was lost. Upon awakening, rats were returned to their cages for the rest of the study.

Thalidomide (THL) Treatment

The THL dose effectively reduced TNF-α synthesis (28). THL (50 mg/kg dissolved in 2% DMSO in saline) or vehicle alone was injected intraperitoneally (0.3 cc vol) into male rats approximately 1–2 h following recovery from ether anesthesia, and daily thereafter at the same time of day, for a total of 5 injections. Rats were euthanized at day 9 ($n = 6$/group) or 21 ($n = 3$/group), 9 total rats per group were assessed for nociceptive sensitivity on days 0, 3, 7 and 9 (for all 9 rats/group). Four groups of three rats/group also were tested on days 13 and 21 as a pilot experiment to determine if latent allodynia or hyperalgesia appeared over the subsequent 12 days.

Anti-TNF-α Antibody Treatment

Male and female Sprague-Dawley rats (200∼225 g) were randomly divided into 4 groups: control + IgG, control + anti-TNF-α, SPS + IgG and SPS + anti-TNF-α (N = 5∼6/group/sex). Anti-TNF-α antibody (30 μg) (29) or the same amount of normal goat IgG were injected during the second hour of restraint in SPS or control rats.

Nociceptive Sensitivity Tests

Rats were assessed changes in nociceptive responses to tactile and thermal stimuli in all groups after placement in clear plastic boxes with a glass floor for thermal tests and a wire mesh floor for tactile assessments. Animals acclimated to the boxes for 15–30 min prior to assessment. A plantar analgesia meter (IITC Life Science Inc., Woodland Hills, CA) was utilized to measure paw withdrawal latency (PWL) to an infrared light beam (thermal sensitivity) directed toward the right hind paw with the lamp set at 25% active intensity. Cut-off time was set at 30 s to prevent tissue damage (30). An Electronic von Frey anesthesiometer (IITC Life Science, Inc., Woodland Hills, CA) was utilized for tactile (mechanical) sensitivity assessment. Paw withdrawal thresholds (PWT) from the von Frey-like stimuli were obtained from the mid-plantar aspect of the right hind paw. The responses to thermal and tactile stimuli were tested 2 h apart. The average of 3 assessments spaced 5 min apart were compared between groups for each test. SPS began at least 1 h after baseline pain thresholds were assessed.

Elevated Plus Maze (EPM) Test

Rats were tested on the EPM on day 9 after SPS for the appearance of anxiety-like behaviors (31). EPM tests occurred between 09:00 and 10:30 h, before nociceptive sensitivity assessment. The plus maze consisted of two open (50 × 10 cm) and two closed (50 × 10 × 40 cm) arms elevated 40 cm above the floor with average light levels 40–55 lux. After placement in the center of the apparatus facing the closed arms, behavior was recorded for 5 min (with the camera focused on the rear 3/4th of the rat's body) and analyzed by Any-maze software (Stoelting Co., Wood Dale, IL). The percentage of open arm entries (number of entries into the open arms divided by total number of entries in both arms), time spent in the open arms, total distance traveled and total time spent immobile were noted. The anxiety index was calculated as described (32), where total exploration on the maze represents the total number of arm entries: Anxiety Index = 1- [[(time spent in open arms/total time on the maze)+(number of entries into open arms/total number of arm entries)]/2], where total time on the maze was 300 sec. Each animal was tested once.

Euthanasia and Sample Collection

Rats were euthanized with Beuthanasia (0.22 mL/kg i.p., Schering-Plough Animal Health, Union NJ). Blood was withdrawn from the heart with an 18-gauge needle (between 15:00 and 17:00 h), placed in Eppendorf tubes and maintained at room temperature for 30 min. Blood samples were then centrifuged at 5,000 × g at 4°C for 5 min, the serum was collected and stored at −80°C. CSF was withdrawn by inserting a 26-gauge needle into the cisterna magna and was immediately stored at −80°C. Brains and spinal cords were extracted and stored at −80°C. Brains were thawed on ice and sliced with a vibratome to dissect hippocampus, hypothalamus, amygdala, prefrontal cortex and periaqueductal gray (PAG) regions according to the Paxinos and Watson rat brain atlas (33) for N/OFQ and TNF-α mRNA and protein quantification.

N/OFQ Quantification

N/OFQ content in sera, CSF and selected brain regions was determined by radioimmunoassay kit (Phoenix Pharmaceuticals, Belmont, CA) as previously described (10). Results are presented as N/OFQ; calculated and expressed in pg/mL for sera and CSF, and pg/mg for hypothalamus and PAG. All samples and standards were assayed in duplicate (50 μL). The sensitivity of the assay was 10 pg/mL and non-specific binding was 2.9%. There was no cross-reactivity with dynorphin A (1–17), enkephalin or β-endorphin.

TNF-α

Blood samples were obtained by tail bleed (200–300 μL) under isoflurane anesthesia or from cardiac puncture. Serum and CSF was collected and prepared as described above. Levels of TNF-α in serum and CSF (50 μL) were quantified using the Rat TNF-α ELISA kit (KRC3011, Invitrogen) as directed by the manufacturer.

Real-Time PCR

TRI reagent (Sigma-Aldrich, MO) was immediately added to dissected tissues for mRNA extraction. cDNA was synthesized using Super-Script III Reverse Transcriptase (Sigma-Aldrich, MO). Real-time PCR was performed using SYBR Green Master Mix (AnaSpec, Fremont, CA) and 125 nM forward and reverse primers (rat TNF-α FWD: 5′-ACCACGCTCTTCTGTCTACTG-3′, REV: 5′-CTTGGTGGTTTGCTACGAC-3′, rat 28S: FWD: 5′-GAAGGCAAGATGGGTCACCA-3′, REV: 5′-GAACTTCCGTGGGTGACTCC-3′, rat GAPDH Fwd: 5′-ACCCAGAAGACTGTGGATGG-3′, Rev: 5′-CAC ATT GGG GGT AGG AAC AC-3′, rat NOP Fwd: 5′-GTT CAA

GGA CTG GGT GTT CAG CCA GGT AGT-3′, rat NOP Rev: 5′-TGC TGG CCG TGG TAC TGT CTC AGA ACT CTT-3′, rat preproN/OFQ Fwd: 5′-TGC ACC AGA ATG GTA ATG TG-3′, Rev: 5′-TAG CAA CAG GAT TGT GGT GA-3′, all from Sigma-Aldrich) in QuantStudio StepOne qPCR (Applied Biosystems). The average of GAPDH and 28S CT values served as an internal standard to which expression of other genes were normalized. Data were analyzed using the comparative Ct method as described (34).

Blood Brain Barrier Permeability

Blood brain barrier permeability was determined by measuring the ratio of CSF albumin to serum albumin. Albumin levels in CSF (100 μL of a 1:500-fold diluted sample) and serum (100 μL of a 1:1,000,000-fold diluted sample) were collected from control or SPS-treated male rats euthanized at 1, 4, and 24 h (32 rats total) after being placed in isolation in their cages following recovery from ether anesthesia. Samples were analyzed by ELISA (GB0032, GenWay Biotech, Inc. San Diego, CA) based on manufacturer's instructions. The standard curve ranged from 0 to 200 ng/ml. Levels of CSF albumin in four rats (three control, one 4 h SPS) were outside the range of detection. With an insufficient volume of CSF remaining to re-assay, the albumin CSF:serum ratio for those rats could not be determined.

Statistical Analysis

Results of D'Agostino & Pearson omnibus normality tests determined if subsequent data analysis should utilize parametric or non-parametric approaches. Outliers were identified by ROUT. Data were analyzed by one- or two-way ANOVA with Tukey's *post-hoc* analysis, as indicated. Non-parametric data were analyzed using the Kruskal-Wallis test. Results were considered significantly different if $p < 0.05$. Analysis was performed with Prism v. 9.2 for Windows (GraphPad Software, Inc.).

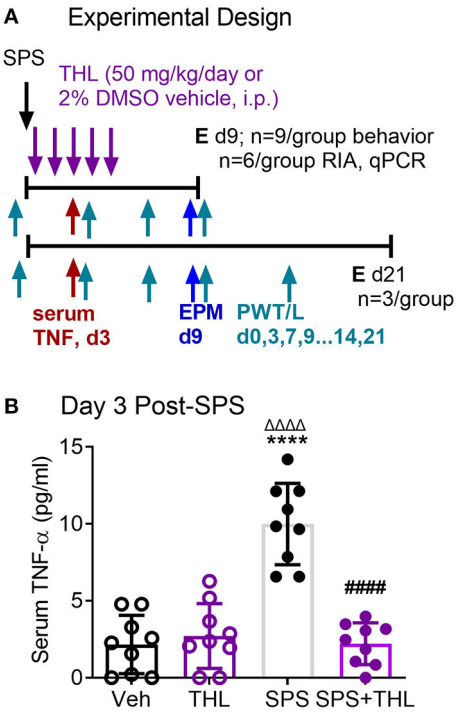

FIGURE 1 | Thalidomide (THL) prevents TNF-α increase in serum of SPS rats. **(A)** Experimental design includes four groups [Vehicle control (Veh), thalidomide (THL), SPS+Veh (SPS) or SPS+THL], with 9 rats/group, 6 rats/group were euthanized (E) at day 9 and 3 in each group were euthanized at day 21. Nociceptive and anxiety-like behavior testing days are noted with arrows and the designations of PWT/L and EPM, respectively. **(B)** Blood was withdrawn from the tail vein on day 3 and serum THL levels were determined by ELISA. Data are presented as mean ± 95% confidence interval and were analyzed by 2-way ANOVA. Significant interaction between THL treatment and stress was noted [$F_{(1, 32)} = 44.64$, $P < 0.0001$], as well as significant effects of treatment and stress, *post-hoc* comparisons were made using Tukey's multiple comparison test ($p < 0.0001$ from Veh****, THL$^{\Delta\Delta\Delta\Delta}$, or SPS$^{\#\#\#\#}$).

RESULTS

THL Prevents SPS-Induced Increase in Serum TNF-α

We previously reported on preliminary studies that prior to appearance of anxiety-like behaviors at day 9, allodynia was evident as early as 3 days post-SPS, and was accompanied by elevated serum TNF-α levels in SPS-treated rats (35). Since that preliminary report, Sun et al. reported hyperalgesia within 1 day of SPS and increased TNF-α in hippocampus 1 day post-SPS (13, 14). As TNF-α can produce hyperalgesia, increase anxiety-like behaviors and increase N/OFQ expression, we hypothesized that blockade of the TNF-α surge would prevent or reduce the TNF-α increases and symptoms noted following exposure to SPS. Thalidomide (THL) blocks synthesis and release of TNF-α in the brain and periphery, so male rats subjected to SPS and their controls were treated with vehicle or an FDA-approved small molecule blood-brain barrier permeable TNF-α synthesis inhibitor (36). Vehicle control and SPS-treated rats received five single daily doses of THL or veh that were initiated immediately following SPS (designated day 1) and continued through day 5 (**Figure 1A**). To confirm the efficacy of THL treatment to inhibit TNF-α synthesis, blood samples were collected by tail bleed on day 3 of SPS. TNF-α levels in serum obtained from those samples was quantified by ELISA (**Figure 1B**) and analyzed by 2-Way ANOVA with Tukey's multiple comparisons test. Data analysis revealed a significant interaction between stress and THL treatment [$F_{(1, 32)} = 44.64$, $P < 0.0001$], as well as significant SPS [$F_{(1, 32)} = 33.17$, $P < 0.0001$] and treatment [$F_{(1, 32)} = 35.75$, $P < 0.0001$] effects. Treatment of SPS rats with THL prevented (#$p < 0.0001$) the TNF-α increase observed in SPS-treated rats (*$p < 0.0001$) 3 days post-SPS, but had no effect on TNF-α levels in in non-stressed rats (THL) compared to vehicle-treated controls (Veh), $N = 9$/group.

THL Prevents Development of SPS-Induced Tactile Allodynia and Reduces Thermal Hyperalgesia

Nociceptive sensitivity to tactile (A) and thermal (B) stimuli was assessed prior to SPS (0) and on days 3, 7, and 9 (**Figure 2**, THL

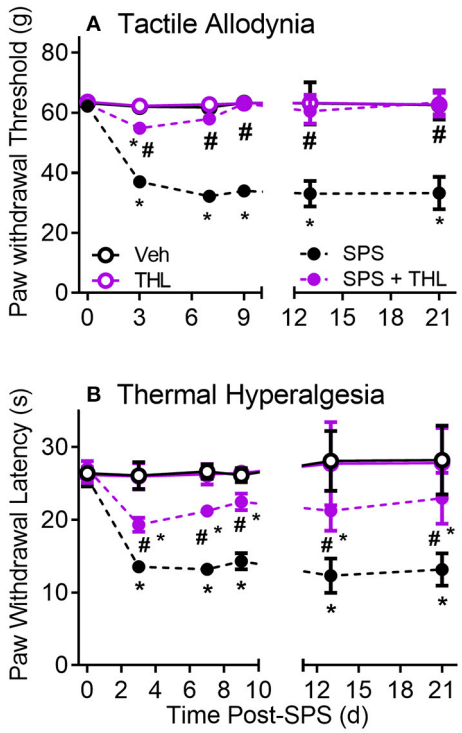

FIGURE 2 | Thalidomide (THL) prevents development of SPS-induced tactile allodynia **(A)** and alleviates thermal hyperalgesia **(B)**. Single daily injections of thalidomide (50 mg/kg, i.p.) from days 1-5 of SPS prevents development of allodynia and reduces thermal hyperalgesia. Sensitivity of THL-treated rats did not differ from that of Veh-treated rats. Results reflect mean ± 95% CI of 9 rats/group through day 9 and 3 rats/group at days 13 and 21, group differs from Veh $^{*}p < 0.0001$, differs from SPS: $^{\#}p < 0.001$.

treatment = days 0–5). Data from day 0 to 9 were analyzed by a 2-Way ANOVA with repeated measures ($N = 9$/group) with Tukey's Multiple Comparison *post-hoc* test. PWT and PWL were significantly reduced in the SPS groups compared to Veh- and THL-treated groups ($^{*}p < 0.0001$). Acute treatment of SPS with THL almost completely prevented the appearance of tactile allodynia ($^{\#}p < 0.0001$) compared to SPS on day 3, by day 7 no differences between SPS+THL, Veh- and THL-treated groups were noted (**Figure 2A**). THL treatment alone had no effect on baseline nociceptive sensitivity. THL also significantly alleviated thermal hyperalgesia in SPS rats (**Figure 2B**), however the effect of THL-treatment on thermal sensitivity was only partially effective as SPS+THL-treated rats continued to exhibit hyperalgesia (albeit less than SPS rats), throughout the 9 or 21 day periods (**Figure 2B**). Data analysis revealed a significant interaction between time and treatment for both tactile [$F_{(9, 96)} = 306.4$, $P < 0.0001$] and thermal [$F_{(9, 96)} = 40.49$, $P < 0.0001$] sensitivity. Significant effects of THL treatment on tactile [$F_{(3, 32)} = 2912$, $P < 0.0001$] and thermal sensitivity [$F_{(3, 32)} = 250.5$, $P < 0.0001$] and of time: tactile [$F_{(3, 96)} = 494.2$, $p < 0.0001$] and thermal sensitivity [$F_{(3, 96)} = 99.98$, $p < 0.0001$], were noted. To confirm that nociceptive sensitivity in THL-treated SPS rats did not gradually return to SPS levels over time, 3 rats per group also were assessed for nociceptive sensitivity on days 13 and 21 (**Figure 2B**). Sensitivity remained as it had been on day 9, with no changes over time. Two-way analysis of PWT and PWL for days 13 and 21 revealed a significant effect of treatment for tactile [$F_{(3, 8)} = 274.9$, $p < 0.0001$] and thermal [$F_{(3, 8)} = 78.09$, $p < 0.0001$] sensitivity.

THL Prevents SPS-Induced N/OFQ Increases in Serum

The hypothesis that the early increase in serum TNF-α following SPS led to allodynia and hyperalgesia, as well as increases in central and circulating N/OFQ levels, was supported by a report that TNF-α increased N/OFQ expression (37). Therefore, the ability of acute THL treatment to prevent subsequent increases in N/OFQ post-SPS were determined in serum and CSF samples obtained from rats euthanized at day 9 and 21 post-SPS (**Figure 3**).

SPS increased serum N/OFQ levels compared to Veh- ($^{****}p < 0.0001$) or THL treatment alone ($^{\Delta\Delta\Delta\Delta}p < 0.0001$), and THL treatment blocked SPS-induced increases in serum N/OFQ ($^{\#\#\#\#}p < 0.0001$), as determined by two-way ANOVA (**Figure 3A**). There was a significant interaction effect between SPS and THL on serum N/OFQ levels [$F_{(1, 20)} = 19.23$, $P = 0.0003$]. Significant SPS [$F_{(1, 20)} = 29.49$, $P < 0.0001$] and treatment effects [$F_{(1, 20)} = 20.92$ $P = 0.0002$] also were observed.

Hemoglobin adulteration of CSF samples from 4 rats in the Veh-treated control group and 1 rat from each of the other 3 day 9 groups precluded assay. Since there were no differences between d9 and d21 vehicle-treated group CSF N/OFQ samples (as determined by unpaired student's *t*-test), they were grouped together to obtain sufficient sample size for analysis. A significant effect of stress was observed [**Figure 3B**, $F_{(1, 16)} = 30.29$, $p = 0.0001$]. SPS increased CSF N/OFQ levels compared to Veh- ($^{***}p < 0.001$) and THL-treated groups ($^{\Delta\Delta}p < 0.01$). Transient THL treatment lowered the SPS-induced increase in CSF N/OFQ levels, but SPS+THL still differed significantly from Veh ($^{*}p < 0.05$). THL treatment did not alter baseline N/OFQ levels when compared to vehicle-treated controls from CSF or serum.

Since SPS also increased N/OFQ levels in the PAG at day 9-post SPS (35), N/OFQ-immunoreactivity was quantified in PAG from rats subjected to SPS in the presence and absence of Veh- or THL-treatments, and euthanized at day 9 (**Figure 3C**). There was a significant effect of SPS on PAG N/OFQ [$F_{(1, 19)} = 4.442$, $p = 0.0486$] as determined by two-way ANOVA ($N = 5$–6/group) (**Figure 3C**), but no *post-hoc* differences were found.

SPS Reduced TNF-α mRNA in Hippocampus and Prefrontal Cortex

The hippocampus (HC), amygdala (AMY), and prefrontal cortex (PFC) process responses to traumatic stress and pain, and express both the NOP receptor and N/OFQ. N/OFQ mRNA in PAG and HC, and NOP mRNA in AMY and PAG were elevated at day 21 post-SPS (17), but earlier time points have not been assayed. Messenger RNA from HC, AMY, and PFC was isolated from rats in Veh, THL, SPS, and SPS+THL groups euthanized at day 9 post-SPS. Changes in TNF-α,

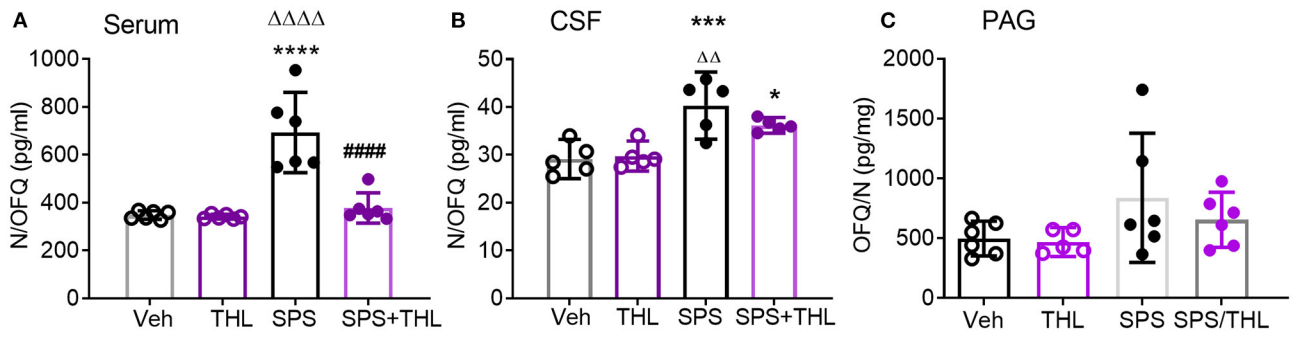

FIGURE 3 | THL reduces SPS-induced increases in serum **(A)** and CSF N/OFQ **(B)**, but not PAG **(C)**. Data are presented as scatter plots with mean ± 95% CI. N/OFQ levels increased in serum (**A**, $^{****}p < 0.0001$) and CSF (**B**, $^{***}p < 0.001$) following SPS, THL treatment reversed N/OFQ increase in both serum and CSF ($^{####}p < 0.0001$) as determined by two-way ANOVA with Tukey's multiple comparisons *post-hoc* tests. SPS also differed from THL ($^{\Delta\Delta\Delta\Delta}p < 0.0001$, $^{\Delta\Delta}p < 0.01$). **(C)** A significant effect of SPS exposure on N/OFQ levels in PAG was observed [$F_{(1, 19)} = 4.442$, $p = 0.04$], but no *post-hoc* differences were found. Rout outlier test excluded one rat from THL **(C)**, $Q = 1\%$.

prepronociceptin and NOP receptor mRNA expression in those brain regions were determined using qPCR (**Figures 4A–F**). TNF-α mRNA levels were reduced in SPS and SPS+THL groups in HIP [**Figure 4A**: $^{*}p < 0.05$ by $F_{(3, 15)} = 4.705$, $p = 0.0165$] and prefrontal cortex [CTX, **Figure 4D**: $^{*}p < 0.05$, $^{**}p < 0.01$, $F_{(3, 12)} = 5.206$, $p = 0.0156$] compared to VEH. Decreased TNF-α mRNA is consistent with compensatory down-regulation following gene activation. Serum and CSF samples from day 9 rats also were assayed for TNF-α levels by ELISA, using a 2-way ANOVA. There was a significant difference between groups for CSF $F_{(1, 16)} = 8.960$, $p = 0.0086$, but no *post-hoc* differences were noted. No significant differences in TNF-α between treatment groups for serum were found (data not shown).

Though no differences were noted for N/OFQ in HIP (**Figure 4B**), a significant difference between groups was noted with N/OFQ mRNA for prefrontal cortex (**Figure 4E**), [$F_{(3, 17)} = 3.969$, $p = 0.0259$], with an increase in PNOC noted in the THL+SPS group compared to Veh alone ($\#p < 0.05$).

No changes in NOP receptor mRNA were noted in HIP (**Figure 4C**). Kruskal-Wallis analysis found a significant difference in group means (**Figure 4F**, $^{*}p = 0.0351$), but no *post-hoc* differences were found. No changes in TNF-α, PNOC or NOP receptor mRNA were found in AMY (data not shown). These changes are consistent with differential regional modulation of NOP receptor peptide and receptor by TNF-α.

THL Effects on Anxiety-Like Behaviors

Since acute THL treatment prevented SPS-induced increases in serum TNF-α, nociceptive sensitivity and modulated NOP receptor and peptide mRNA, its effect on the development of anxiety-like behaviors was assessed in Veh-, THL-, SPS-, and SPS+THL-treated rats using the EPM test (**Figure 5**). SPS was a significant factor in decreased number of open arm entries and in the increased anxiety index (**Table 1**), consistent with previous studies (10, 17). However, *post-hoc* analyses did not confirm that SPS groups differed from Veh-treated controls in any parameter shown. THL treatment was a significant factor in *all* parameters assessed (**Table 1**) and *post-hoc* tests revealed that rats receiving THL-treatment alone differed from Vehicle- ($^{*}p < 0.05$) or SPS-treated rats ($\#p < 0.05$), making it difficult to interpret the impact of THL treatment on anxiety-like symptoms produced by SPS.

BBB Permeability 1–24 h Post-SPS

The blood-brain barrier (BBB) acts a selective physical barrier between the CNS and the periphery whereby it regulates the transport of molecules between both compartments (38). While albumin is present in high amounts in serum, it is normally excluded from CSF due to its high molecular weight. BBB disruption in humans is often measured by determining the ratio of albumin in a subject's CSF compared to levels in serum ($\text{Albumin}_{\text{CSF : serum}}$ ratio) (39). Elevated ratios indicate BBB disruption (40–43). Traumatic stress was found to produce acute, transient increases in BBB permeability (44). Therefore, to determine if SPS alters BBB permeability, CSF and serum samples were recovered from SPS or control rats euthanized at 1, 4, and 24 h following recovery from ether anesthesia (**Figure 6**), and albumin levels determined by ELISA. Data from control rats from each time point comprised the control group. Levels of CSF albumin in three control and one 4-h SPS-treated rats were outside the level of detection, thus the CSF:serum ratio for those rats could not be determined. One rat from the 4 h group was determined to be an outlier by the ROUT ($Q = 1\%$), and was excluded. Because three of the four groups failed the Shapiro & Wilk normality test, analysis was performed using the Kruskal-Wallis test. No significant differences between groups were found, consistent with an intact BBB following SPS.

Changes in Circulating TNF-α and TNF-α mRNA 1–24 h Post-SPS

While levels of TNF-α in serum at 3 days post-SPS were higher than in untreated rats, they were still lower than would be expected immediately following exposure to a traumatic stress. Serum TNF-α often increases shortly after an injury or stressful event, and it was likely that the day 3 time point represented the

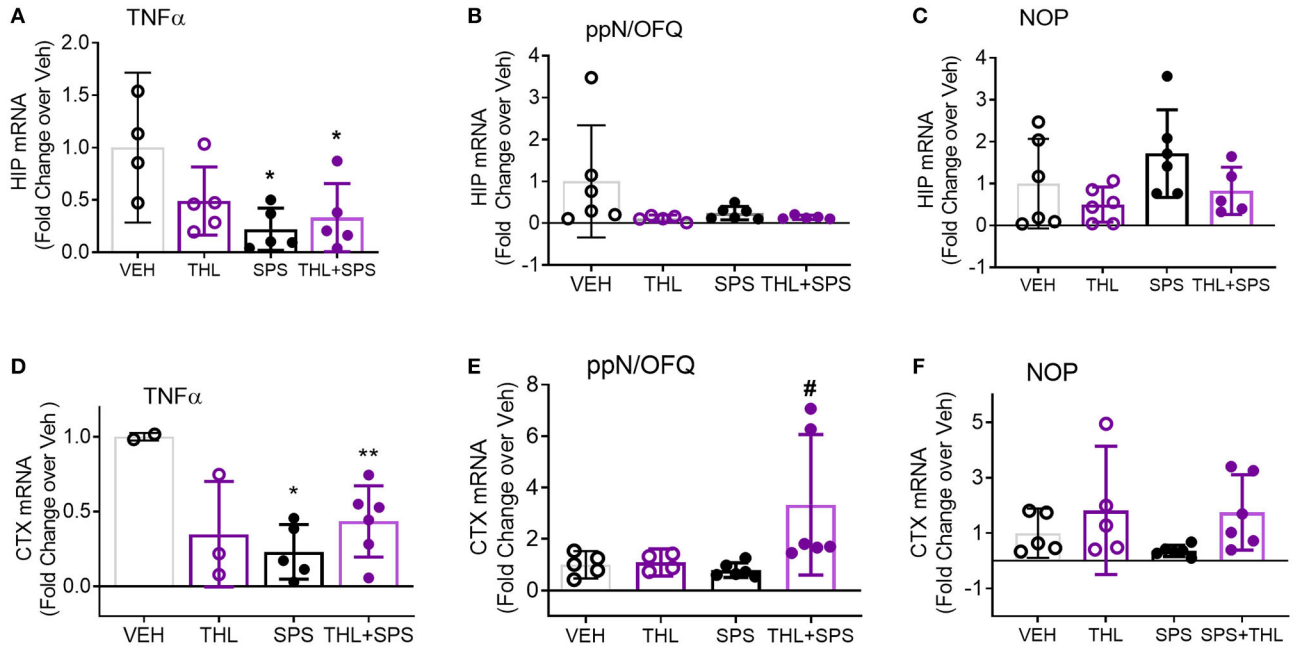

FIGURE 4 | TNF-α mRNA expression declines in hippocampus (HIP) and prefrontal cortex (CTX) with SPS. Messenger RNA from HIP and CTX was isolated from tissues frozen immediately after extraction on day 9. Quantitative pcr (qPCR) was performed to determine changes in TNF-α, prepronociceptin (ppN/OFQ), and NOP receptor mRNA expression in all four groups. The ratio of each sample to GAPDH/28S was normalized to the mean of vehicle control to determine fold change in mRNA with treatment. Data were analyzed by one-way ANOVA with Tukey's multiple comparisons test **(A–C,E)** or Kruskal-Wallis **(B,F)**. Purity of mRNA from one Veh and one THL rat were below the threshold for use (0.8). Rout outlier test exclusions include 1 THL+SPS **(A)**, 1 SPS and 1 THL+SPS **(B)**, 1 THL+SPS **(C)**, and 1 THL **(E)**. TNF message was below the level of detection for 1 Veh **(A)**, and 3 Veh, 2 THL, and 1 SPS **(D)**.

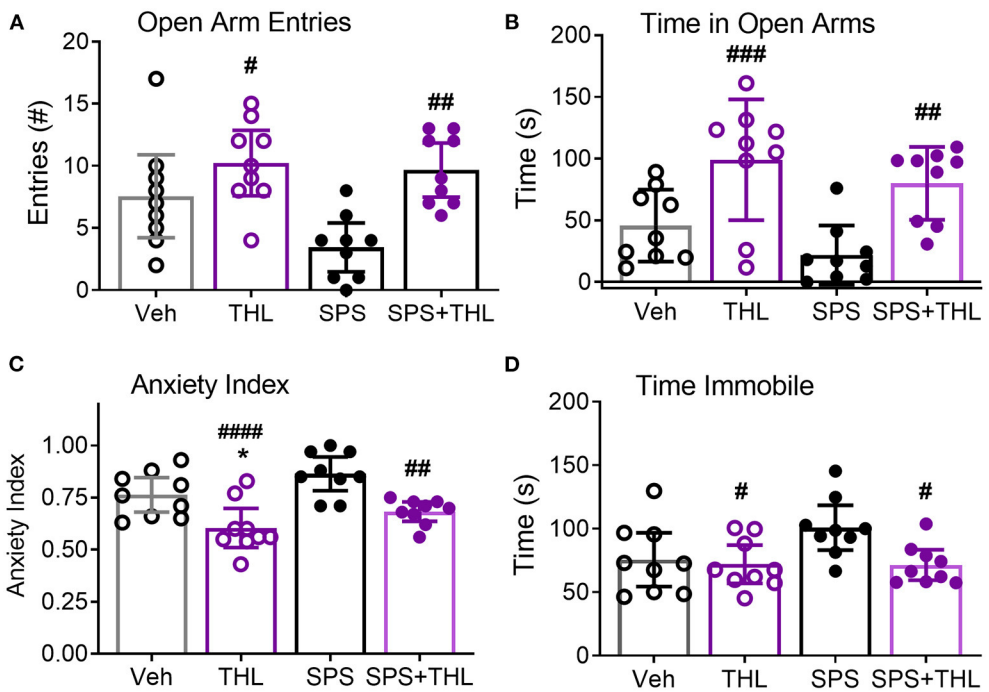

FIGURE 5 | THL modulates anxiety-like behaviors in vehicle-treated control and SPS rats. Results of four different parameters are shown: Open arm entries **(A)**, Time in open arms **(B)**, Anxiety index **(C)**, and Time immobile **(D)**. Data are represented as scatter plots of $N = 9$/group, with 95% CI. *Post-hoc* analysis indicates significant effects of THL treatment alone: differs from Veh-treated ($^{*}p < 0.05$) and SPS ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$.).

TABLE 1 | ANOVA results of anxiety-like behaviors from THL and anti-TNF-α antibody treatment studies using EPM.

Parameter	Factor	$F_{(DFn, DFd)}$	*P*-value
Thalidomide-SPS EPM			
Open arm entries	Interaction	$F_{(1, 32)} = 2.528$	0.1216
	SPS	$F_{(1, 32)} = 4.356$	**0.0449**
	THL	$F_{(1, 32)} = 15.84$	**0.0004**
Time in open arms	Interaction	$F_{(1, 32)} = 0.04559$	0.8323
	SPS	$F_{(1, 32)} = 3.530$	0.0694
	THL	$F_{(1, 32)} = 23.83$	**<0.0001**
Anxiety index	Interaction	$F_{(1, 32)} = 0.1070$	0.7457
	SPS	$F_{(1, 32)} = 7.021$	**0.0124**
	THL	$F_{(1, 32)} = 25.05$	**<0.0001**
Time immobile	Interaction	$F_{(1, 32)} = 3.137$	0.0860
	SPS	$F_{(1, 32)} = 2.847$	0.1012
	THL	$F_{(1, 32)} = 5.103$	**0.0308**
α-TNFα Treatment—EPM	**Sex/Panel**		
Open arm time	Males (A)	$F_{(2, 18)} = 8.366$	**0.0027**
	Females (D)	$F_{(2, 19)} = 2.319$	0.1255
Anxiety index	Males (B)	$F_{(2, 19)} = 6.494$	**0.0071**
	Females (E)	$F_{(2, 20)} = 0.7449$	0.4875
Time immobile	Males (C)	$F_{(2, 19)} = 0.7839$	0.4708
	Females (F)	$F_{(2, 20)} = 0.5612$	0.5793

*Bold font represents that the factor was significant in the thalidomide-SPS study (**Figure 4**), or that there was a significant difference between groups for each of the panels in the anti-TNF-alpha SPS study (**Figure 11**).*

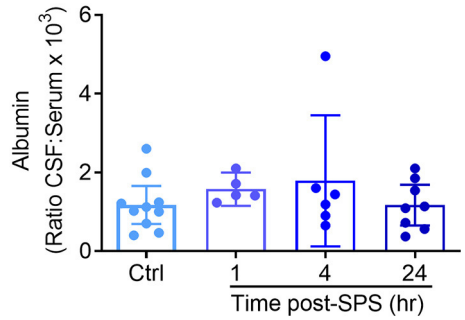

FIGURE 6 | No evidence that SPS disrupts blood brain barrier permeability between 1 and 24 h post-SPS. The ratio of albumin in the CSF and serum from rats euthanized at each time point post-SPS was determined by ELISA and is presented as a scatter plot with mean ± 95% CI. No differences in the CSF to serum albumin ratio were found between groups ($p = 0.3571$) by Kruskal-Wallis test.

trailing end of an earlier surge in TNF-α levels. Thus, serum TNF-α from rats in Ctrl, 1, 4, and 24 h post-SPS groups was determined by ELISA (**Figure 7A**). As anticipated, a significant difference between groups was noted [$F_{(2, 27)} = 379.3$, $P < 0.0001$] using one-way ANOVA with Tukey's multiple comparison test. Serum TNF-α levels increased 4 and 24 h after SPS compared to the control group ($^{***}p < 0.001$, $^{****}p < 0.0001$). The 24 h post-SPS group also differed significantly from the 4 h post-SPS group ($^{\#\#\#\#}p < 0.0001$), confirming that levels began to decline after 4 hr. Both 4 and 24 h time points (**Figure 7A**) were higher than levels noted 3 days post-SPS (**Figure 1B**). TNF-α levels were not elevated in the serum at 1 h, therefore TNF-α mRNA levels were quantified from two potential tissue sources of circulating TNF-α (peripheral blood cells and spleen), 1 h post-SPS (**Figure 7B**). TNF-α mRNA levels did not differ in spleen between groups, but TNF-α mRNA increased 5-fold in circulating blood cells isolated from SPS rats ($^{*}p < 0.05$) as determined by unpaired *t*-test.

Effects of Anti-TNF-α Antibody Treatment on Development of Allodynia and Hyperalgesia in Male and Female Rats Following SPS

To support the hypothesis that circulating TNF-α released 1–4 h post-SPS resulted in SPS-induced allodynia, hyperalgesia and anxiety-like behaviors, male and female SD rats were divided into one of four groups: Sham Control + normal rat serum (Ctr/IgG), Sham Ctr + anti-TNF-α antibody (Ctr/α-TNF-α), SPS+IgG and SPS + α-TNF-α. Three hundred μl (30 μg) IgG or anti-TNF-α was injected into the tail vein (i.v.) during the last few min of the 2 h period of restraint in step one of the SPS protocol (**Figure 8**). Four hour post-SPS, rats were anesthetized with isoflurane and ∼250 μl of blood volume was withdrawn from the tail vein to assay for serum TNF-α. Unfortunately, none of the assays (males or females) detected serum TNF-α (2, 4, or 24 h), perhaps because the IgG interfered with the assay.

Assessment of sensitivity to tactile and thermal stimuli occurred on days 1, 3, 7, and 9 (for females) post-SPS, and assessment of anxiety-like behaviors took place on the morning of day 9, rats were euthanized on day 9 following EPM assessment. As anticipated, SPS produced tactile allodynia and thermal hyperalgesia in males (**Figures 9A,B**, purple symbols) and females (**Figures 9C,D**, fuchsia symbols). Treatment of SPS rats with the anti-TNF-α antibody (SPS+α-TNF-α antibody) protected them almost completely from developing allodynia and hyperalgesia, similar to rats receiving THL. Data were analyzed by 2-way ANOVA with Tukey's multiple comparisons test, revealing a significant effect of anti-TNF antibody treatment on tactile allodynia [$F_{(3, 72)} = 7.640$, $p = 0.0002$] and a significant interaction between treatment and time for thermal hyperalgesia in males [$F_{(9, 72)} = 4.753$, $p < 0.0001$]. Significant effects of time and treatment also were noted for thermal hyperalgesia in males. For females, data analysis revealed significant interactions between anti-TNF-α antibody treatment and time for tactile allodynia [$F_{(12, 98)} = 1.897$, $p = 0.0437$] and thermal hyperalgesia [$F_{(12, 98)} = 2.224$, $p = 0.0160$]. Increased sensitivity in an SPS+anti-TNF-α group was noted only to thermal stimuli in female rats at day 7, but it was back to baseline by day 9 (**Figure 9D**). Therefore, the single injection of anti-TNF-α antibody shortly after traumatic stress prevented development of allodynia and hyperalgesia in male and female rats.

Effects of Anti-TNF-α Antibody Treatment on N/OFQ Levels in Male and Female Rat Brain and Serum

There were no differences in N/OFQ levels between Ctr+IgG and Sham+α-TNF-α, so data from those groups were pooled (designated as Ctr/α-TNF-α) for male and female serum

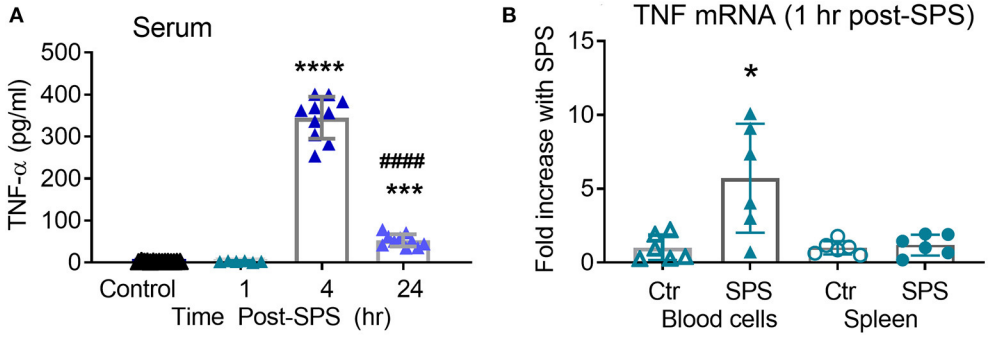

FIGURE 7 | Serum TNF-α increases 4–24 h post-SPS. Serum samples collected for blood brain barrier permeability also were assayed for TNF-α levels by ELISA **(A)**, data presented as scatter plot with mean ± 95% CI ($N = 6$–16/group). TNF-α mRNA **(B)** increased in circulating blood cells ($p < 0.05$), but not spleen, 1 h post-SPS as determined by unpaired student's *t*-test. Real-time qPCR was performed as described above, normalized to mean $2^{\Delta CT}$ of control rats to get fold change ($n = 6$ per group).

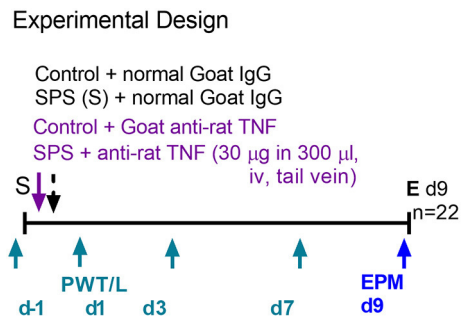

FIGURE 8 | Experimental design for anti-TNF-α antibody treatment in males. Female design was the same except nociceptive sensitivity assessments also were made on d9 ($n = 24$ for female rats).

(**Figures 10A–D**). SPS increased N/OFQ levels in male CSF [$F_{(2, 18)} = 3.90$, $p = 0.0392$], PAG [$F_{(2, 19)} = 4.522$, $p = 0.0248$] and hypothalamus [$F_{(2, 19)} = 6.853$, $p = 0.0057$] post-SPS as determined by one-way ANOVA (**Figures 10A,C**). Since TNF-α can increase N/OFQ mRNA and peptide, we posited that increased TNF-α with SPS leads to increased synthesis and release of N/OFQ that contributes to SPS-induced allodynia, hyperalgesia and anxiety-like behaviors. One-way ANOVA reveals that the anti-TNF-α antibody treatment prevented increased N/OFQ levels in serum ($^{\#}p < 0.05$), and HYPO in males ($^{\#}p < 0.05$) (**Figures 10B,D**). CSF samples are more difficult to obtain from female rats. Unfortunately, all CSF samples assayed from female rats were outside the range of the RIA kit except for SPS, and there was insufficient volume of CSF to repeat the assay. No differences between treatment groups were noted with serum from female rats collected at day 9 (**Figure 10D**, $p = 0.2203$).

However, PAG and HYP from female rats exhibited the same SPS- and α-TNFα treatment induced changes in N/OFQ as males (**Figure 10E**). Female PAG Ctr and α-TNFα alone groups did differ by *t*-test, so those groups were not pooled for either brain region in **Figure 10E**. For PAG, there was a significant effect of SPS [$F_{(1, 20)} = 8.507$, $p = 0.0085$] and of α-TNFα treatment [$F_{(1, 20)} = 8.706$, $p = 0.0079$]. N/OFQ levels in PAG from SPS rats differed significantly from Ctr + α-TNFα ($^{\Delta\Delta}p = 0.01$). For female hypothalamic N/OFQ, there was a significant effect of SPS [$F_{(1, 20)} = 10.45$, $p = 0.0042$] and of α-TNFα [$F_{(1, 20)} = 10.92$, $p = 0.0035$]. SPS differed from control ($^{**}p < 0.01$), Ctr + α-TNFα ($^{\Delta\Delta\Delta}p < 0.001$), and from SPS + α-TNFα ($^{\#\#}p < 0.01$).

Comparisons between male and female N/OFQ between serum, PAG and HYP were performed by 2 way ANOVA. For serum N/OFQ, no interaction between sex and treatment group was found, but there were significant effects of sex [$F_{(1, 40)} = 8.139$, $p = 0.0068$] and of group [$F_{(2, 40)} = 4.325$, $p = 0.0199$]. No *post-hoc* differences between males and females were noted within any treatment group. Similar results were obtained when analyzing N/OFQ levels in the PAG, with effects of sex [$F_{(3, 38)} = 9.539$, $p = 0.0037$] and group [$F_{(3, 38)} = 7.466$, $p = 0.0005$] but no differences between males and females within each group. In the hypothalamus, no differences were noted with sex as a variable, but there was a significant group effect [$F_{(3, 38)} = 12.14$, $p < 0.0001$].

Effects of Anti-TNF-α Antibody Treatment on N/OFQ and NOP Receptor mRNA in Male and Female Rat Brain Regions

Levels of ppN/OFQ and NOP receptor mRNA from HYP, PAG, prefrontal cortex (CTX) and amygdala in male and female rats treated with or without anti-TNF-α antibody and SPS were quantified by qPCR to determine if circulating TNF-α contributes to changes noted with SPS. SPS-induced increases in ppN/OFQ mRNA in the female PAG (**Table 2**) that paralleled increased N/OFQ peptide in that brain region (**Figure 10E**), but unlike the peptide, α-TNFα treatment reversed SPS-induced 2.4-fold increase in ppN/OFQ mRNA levels ($^{\#\#}p < 0.01$). Similar effects were found with ppN/OFQ mRNA in female CTX (reversal of 1.8-fold mRNA increase). In the male HYP, SPS + α-TNFα treatment significantly increased ppN/OFQ mRNA compared to SPS ($^{\#}p < 0.05$). Despite increased N/OFQ peptide in male PAG with SPS (**Figure 10D**), ppN/OFQ mRNA levels did not

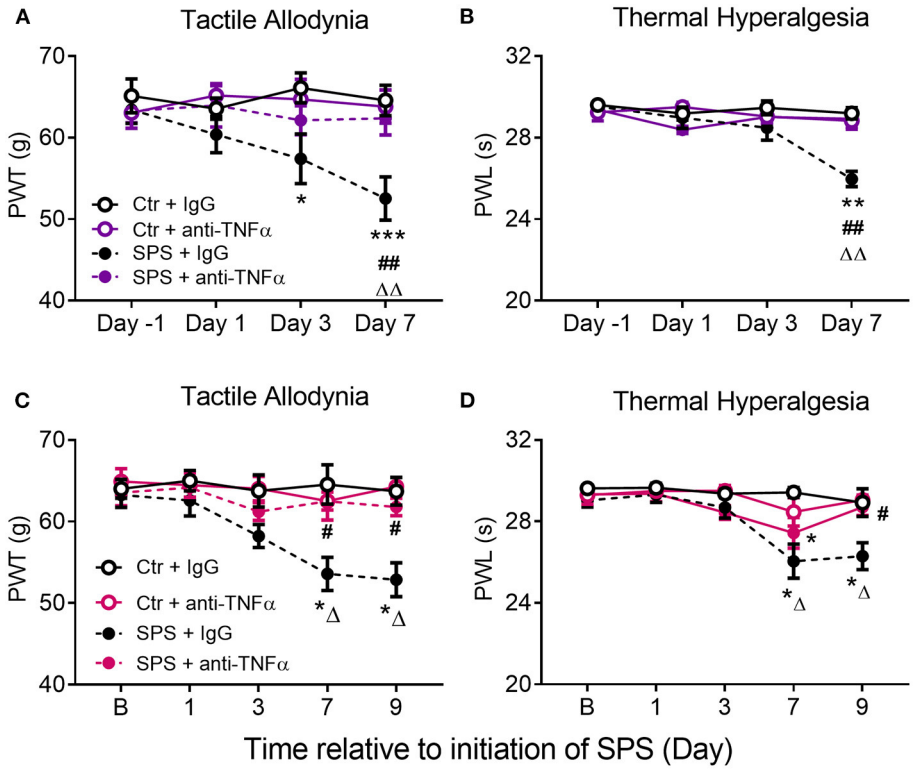

FIGURE 9 | Anti-TNF-α antibody treatment prevents development of SPS-induced tactile allodynia **(A,C)** and thermal hyperalgesia **(B,D)** in male **(A,B)** and female **(C,D)** rats. Rats received a single injection of goat-anti rat TNF-α diluted in saline or the same volume of goat IgG during the SPS procedure. Data are presented as mean ± SEM of 5–6 rats per group. Data were analyzed by two-way ANOVA with Tukey's multiple comparison test. Significant differences were noted as follows: from control: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; from Ctr+anti-TNFα: $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$; and from SPS: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

differ from controls in that region (**Table 2**). No other changes in N/OFQ mRNA were noted.

SPS increased NOP receptor mRNA in PAG of female rats ($^{*}p < 0.05$) and in the HYP of male rats ($^{*}p < 0.05$), but this increase was not blocked by anti-TNF antibody treatment in either case (**Table 2**). No other changes in NOP mRNA were found in the other regions tested in males or females.

Effects of Anti-TNF-α Antibody Treatment on SPS-Induced Anxiety-Like Behaviors in Male and Female Rats

Though not chosen as a primary endpoint, the robust nature of the traumatic stress on anxiety-like behaviors produced very interesting results (**Figures 11A–F**). As previously reported by others and us, SPS rats exhibited a number of anxiety-like behaviors 9 days post-SPS. Time in open arms was significantly reduced in SPS/IgG male rats ($^{**}p < 0.01$, **Figure 11A**) and anxiety index increased ($^{**}p < 0.01$; **Figure 11B**), consistent with elevated anxiety-like behaviors. Anti-TNF-α antibody treatment prevented the SPS-induced decreased time in open arms ($^{\#\#}p < 0.01$, **Figure 11A**), but did not quite reverse the elevated anxiety index, $p = 0.09$ (**Figure 11B**). No significant differences between groups were noted for any parameter for female rats (**Figures 11D–F**); no differences in immobile time were noted for males or females (**Figures 11C,F**).

DISCUSSION

This paper demonstrates, for the first time, the time course of circulating TNF-α following trauma in a preclinical model of PTSD (SPS). It also shows that that blockade of TNF-α synthesis or action prevented the development of SPS-induced allodynia and alleviated the development of hyperalgesia in male and female rats, as well as prevented upregulation of N/OFQ in serum of male rats, and in the HYP of male and female rats.

The pathophysiology of co-morbid PTSD and chronic pain is unclear. Patients with PTSD often exhibit excessive inflammatory activities of the immune system including increased circulating pro-inflammatory cytokines, however few studies have tested the relationship between inflammatory cytokines, PTSD and pain experimentally in preclinical models. Previous work in our lab and others has shown that the SPS preclinical model for PTSD induces the development of persistent allodynia, thermal hyperalgesia, visceral sensitivity and increased anxiety-like behaviors (10–14, 17, 18). Accompanying these exaggerated pain responses are increased N/OFQ levels in both serum and CSF, and an increase in circulating pro-inflammatory cytokine

TABLE 2 | Effects of α-TNF antibody treatment on SPS-induced changes in ppN/OFQ and NOP mRNA from Day 9 post-SPS and control rats.

	ppN/OFQ mRNA (fold Change over Ctr)			
Group	**Ctr**	**SPS/IgG**	**SPS/α-TNFα**	**ANOVA**
Females				
Brain region				
PAG	1.0 ± 0.5	**2.4** ± 1.2**	**1.1 ± 0.5##**	***P* = 0.0019**
HYP	1.0 ± 0.5	1.1 ± 0.6	0.8 ± 0.4	*P* = 0.6596
AMY	1.0 ± 0.7	0.5 ± 0.9	0.9 ± 0.5	*P* = 0.3986
CTX	1.0 ± 0.4	**1.8 ± 1.0***	**1.0 ± 0.3#**	***P* = 0.0186**
Males				
Brain region				
PAG	1.0 ± 0.4	1.0 ± 0.6	1.0 ± 1.5	*P* = 0.9807
HYP	1.0 ± 0.8	0.1 ± 0.6	**1.8 ± 1.6#**	***P* = 0.0218**
AMY	1.0 ± 0.5	1.1 ± 0.3	1.2 ± 0.2	*P* = 0.7088
CTX	1.0 ± 1.2	1.4 ± 0.8	0.8 ± 0.3	*P* = 0.4961
	NOP mRNA (fold change over Ctr)			
Group	**Ctr**	**SPS/IgG**	**SPS/α-TNFα**	**ANOVA**
Females				
Brain region				
PAG	1.0 ± 0.4	**1.9 ± 1.1***	1.2 ± 0.3	***P* = 0.0326**
HYP	1.0 ± 0.6	0.9 ± 0.4	1.0 ± 0.5	*P* = 0.9623
AMY	1.0 ± 0.6	0.9 ± 0.4	1.4 ± 0.6	*P* = 0.2156
CTX	1.0 ± 0.4	1.8 ± 1.3	1.5 ± 0.7	*P* = 0.1129
Males				
Brain region				
PAG	1.0 ± 0.3	3.3 ± 2.9	4.7 ± 6.0	*P* = 0.2475
HYP	1.0 ± 0.5	**2.6 ± 0.9***	2.1 ± 1.5	***P* = 0.0167**
AMY	1.0 ± 1.2	0.6 ± 1.0	1.0 ± 1.0	*P* = 0.7886
CTX	1.0 ± 0.9	1.4 ± 1.0	0.7 ± 0.6	*P* = 0.4001

*Individual values from SPS/IgG and SPS/α-TNFα groups (n = 6 each) were normalized to the mean value of combined Ctr/IgG and Ctr α-TNFα group (n = 12) to determine fold change in mRNA. Differences between groups were determined using one-way ANOVA with Tukey's multiple comparisons post-hoc test. Data are presented as mean ± SD, (*indicates difference from Ctr, # indicates difference from SPS). Bold font highlights significantly different groups.*

TNF-α 3 days after SPS. Elevated TNF-α is in concordance with human data whereby PTSD patients including those with non-combat related trauma were found to have elevated serum TNF-α compared to non-PTSD controls (21, 22). The frequency and severity of PTSD symptoms correlated with several different cytokines including TNF-α (21, 22). To ascertain whether the increase in serum TNF-α levels that occurs acutely post-SPS play a role in the development of SPS-induced nociceptive sensitivity and elevated N/OFQ, a TNF-α synthesis inhibitor, thalidomide, and an antibody targeting rat TNF-α were employed.

TNF-α may arise from peripheral and central sources, producing pro- and anti-inflammatory effects, including transcriptional activation. In the periphery, TNF-α may arise from several sources following severe stress such as spleen, lymph nodes, macrophages, NK cells, CD4+ lymphocytes, neutrophils, mast cells, and eosinophils (25, 45–52). We identified ∼ 5-fold increase in TNF-α mRNA in circulating blood cells 1 h post-SPS (**Figure 7**), consistent with a peripheral TNF-α source acutely.

Besides neurons (19, 53), N/OFQ is expressed in glial cells from rat forebrain and SC, dorsal root ganglia (DRG) (54) and immune cells. Neutrophils secrete N/OFQ concurrent with degranulation, and N/OFQ induces leukocyte chemotaxis [for review see (55)]. The ability of N/OFQ to activate NFκB (56) provides a means for it to regulate cytokine and chemokine production as well.

Inflammatory mediators released by glial cells (TNF-α, CNTF) induce profound up-regulation of N/OFQ mRNA and peptide (19, 37, 53, 57). In the brain, N/OFQ acts as an "anti-opioid", increasing hyperalgesia (58). Nanomole doses of N/OFQ produces analgesia when administered intrathecally (59), however femto-picomole levels of i.t. N/OFQ produce hyperalgesia and touch-evoked allodynia (60). Peripheral N/OFQ also contributes to hyperalgesia, distinct from the secondary hyperalgesic responses it may produce (61, 62). Up-regulation of N/OFQ mRNA in dorsal root ganglion and spinal cord following induction of experimental neuropathic pain was blocked with the microglial/peripheral immune cell inhibitor, minocycline (63). Minocycline also blocked PTSD fear and anxiety-like behaviors shortly after systemic (64) and intra-hippocampal administration (13), suggesting that the inflammatory mediator(s) mediating those actions, such as TNF-α, were produced in the hippocampus as well as peripherally. Spinal cord microglia and astrocytes are key players in pain modulation. They contribute to the initiation and maintenance of persistent pain states as well as provide structural and trophic support for neurons. Activation of microglia and astrocytes in the spinal cord contribute to central sensitization, characterized by the development of allodynia and hyperalgesia to tactile and thermal stimuli after nerve injury or peripheral inflammation (64–66).

Neuroinflammation in the CNS refers to the microglial response, and to a lesser extent that of astrocytes and oligodendrocytes (67). Although astrocytes and neurons are able to produce TNF-α, microglia are the primary source of this cytokine during neuroinflammation (67). During the initial response to acute stress, TNF-α activates corticotrophin releasing factor (CRF)-containing neurons in the hypothalamic paraventricular nucleus (68) resulting in CRF-mediated cortisol release, in order to induce negative feedback to prevent an overshoot of inflammatory processes (55, 69–72). PTSD chronically activates and dysregulates the HPA axis wherein negative feedback is enhanced (73).

The ratio of albumin in CSF compared to serum serves as an index of the integrity of the blood-CSF (BBB) barrier. Increases in this ratio denote increased CSF permeability and thus, decreased BBB integrity. No changes in BBB permeability were detected over the 24 h period following SPS. This is consistent with a role of peripheral TNF-α in the initiation of allodynia and hyperalgesia. Though TNF-α may be transported across the BBB (74), increased BBB permeability would facilitate cytokine infiltration to the CNS. The presence of increased inflammatory mediators, such as TNF-α, leads to activation of microglia and recruitment of peripheral blood monocyte entry into the brain

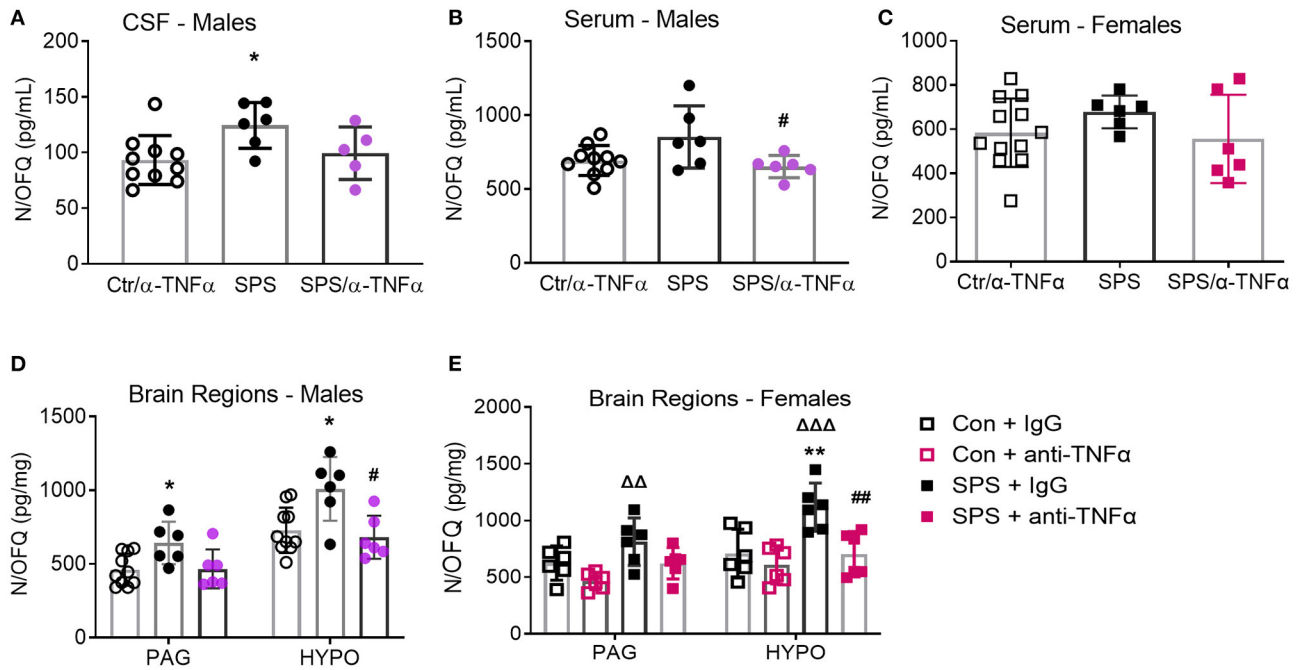

FIGURE 10 | Anti-TNF-α antibody treatment prevents SPS-induced increases in N/OFQ levels in males and females. Data from sham controls receiving IgG and sham controls receiving anti-TNFα antibody injections were combined and compared to SPS+IgG and SPS+anti-TNF-α antibody groups collected on day 9 post-SPS as scatter plots showing mean ± 95% CI. One SPS+anti-TNF antibody rat sample was excluded by Rout **(A)**. SPS treatment increased N/OFQ levels in CSF **(A)**, periaqueductal gray (PAG: **D**) and hypothalamus (HYPO: **D**) in male rats, $^{*}p < 0.05$ as determined by one-way ANOVA compared to sham-treated rats. Antibody treatment prevented N/OFQ increases in the HYPO ($^{\#}p < 0.05$). Though N/OFQ levels were not elevated by SPS in serum **(B)**, N/OFQ levels were significantly lower in serum from SPS + α-TNF-α antibody treated rats than from SPS rats ($^{\#}p < 0.05$). There were no significant differences of serum N/OFQ between groups in females **(C)**. However, SPS-induced increases in female PAG and HYP ($^{\Delta\Delta}p < 0.01$ and $^{\Delta\Delta\Delta}p < 0.001$ from Con+anti-TNFα), and anti-TNFα antibody treatment reversal of increased N/OFQ in HYP ($^{\#\#}p < 0.01$ compared to SPS) **(E)** were the same as those in males **(D)**.

parenchyma that is associated with pain, depressive and anxiety disorders (75, 76).

Thalidomide crosses the BBB (77) to produce several centrally mediated effects (36, 78). Though we did not measure thalidomide levels, the significant effect of THL alone on anxiety-like behaviors, in the absence of traumatic stress, confirms that it penetrates the BBB. THL prevented the development of allodynia and reduced thermal sensitivity by 50%, even for at least 2 weeks after THL administration was discontinued (**Figure 2**). THL is not selective for TNF-α, but inhibits the synthesis of several inflammatory cytokines.

The efficacy of a single administration of a circulating antibody against TNF-α to prevent initiation of allodynia and almost completely alleviate hyperalgesia following SPS in the presence of an intact BBB suggests that the initial surge in serum TNF-α is an integral factor in initiating the pathophysiological cascade of events of SPS. This is in concordance with other experimental models implicating TNF-α with the emergence of pain symptoms and in particular the neuroinflammatory and nociceptive properties that play a role in central sensitization (79, 80). Serum TNF-α levels at 4 and 24 h post-SPS were significantly larger than day 3 post-SPS levels and were comparable to levels found in inflammatory disorders with depressive mood symptoms (81).

Antibodies in general cross the BBB very poorly unless the barrier is leaky to very large proteins, such as with chronic inflammation or brain tumor, or the antibody can target a receptor-mediated transport protein to do so (82). Our data indicates that the blood-brain barrier remained intact following SPS for at least 24 h following SPS (**Figure 6**), consistent with a previous study that examined a variety of stressful stimuli on blood-brain barrier integrity (83). If there was localized disruption following SPS, it was masked by the overall ability of the barrier to prevent albumin from entering the CNS. It is unlikely that significant amounts of the anti-TNF-α antibody crossed the BBB since the barrier appeared intact. Indeed previous use of an anti-TNF-α antibody (i.v.) to treat dental pain in rats, found that its ability to block pain was gone by the third day after i.v. administration (84). This limited time of action would suggest that allodynia and hyperalgesia in the SPS model would NOT have been alleviated on days 5–9 (as it was), if the peripheral source of the pain stimulation (TNF-α) had not been removed. Differences between reversal of N/OFQ levels in PAG and HYP by TNF antibody treatment also may reflect the relatively close proximity of the HYP to the BBB compared to the PAG. Further studies of changes in TNF and N/OFQ mRNA and peptide in those regions at earlier time points may provide clarification.

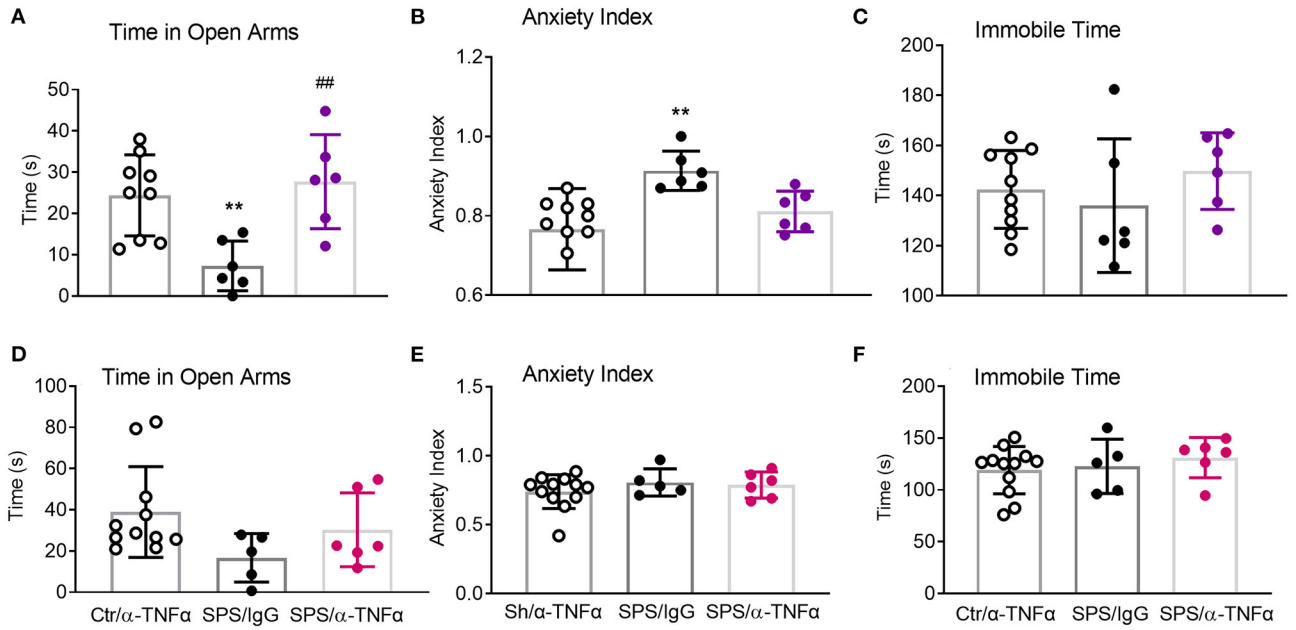

FIGURE 11 | Anti-TNF-α antibody treatment had mixed effects on EPM parameters in males. Time in Open Arms **(A)**, Anxiety Index **(B)**, and Immobile time **(C)** were assessed in males **(A–C)** and females **(D–F)**, respectively. Data from sham controls receiving IgG and sham controls receiving anti-TNFα antibody injections were combined, and compared to SPS+IgG and SPS+α-TNF-α. SPS produced anxiety-like behaviors in male rats **(A,B)**, but not in female rats **(D,E)**. Anti-TNF-α antibody treatment reversed decreased time in open arms (**A**, $^{\#\#}p<0.01$), but not the increased anxiety index **(B)**. Data are presented as scatter plots, showing mean ± SD.

One limitation of the CSF:Serum albumin ratio method is that it does not directly measure the location and physical extent of BBB disruption, and small disruptions of the barrier may be masked. Additional methods may be useful to fully ascertain the sites and impact of stress/trauma on BBB integrity in this model in the future (85–88).

Of particular interest to this study is (1) previous evidence that TNF-α increased prepronociceptin mRNA and N/OFQ peptide (19), and that (2) N/OFQ activates NFκB through the NOP receptor (56). N/OFQ also appears to play a role in increased N/OFQ since blockade or loss of NOP prevents N/OFQ up-regulation at later time points as well (17, 18). Upregulated signaling by the neuropeptide Nociception/Orphanin FQ (N/OFQ)- NOP receptor complex downstream of TNF-α appears to sustain chronic pain and PTSD symptoms. N/OFQ levels are elevated in serum and cerebrospinal fluid (CSF) of patients with other forms of chronic pain, which is not surprising since supraspinal N/OFQ may increase pain sensitivity by inhibiting the descending analgesic pathway.

We previously reported that SPS increased levels of N/OFQ in the CSF and brain on days 9–28, and blockade or loss of NOP receptor prevented this increase in male rats. In the current study, serum N/OFQ levels also significantly increased by day 9 of SPS, but only in male rats. THL blocked increased N/OFQ in serum and CSF, correlating with alleviation of TNF-α-induced allodynia and hyperalgesia. Though the results with anti-TNF-α antibody treatment appear more nuanced, they reflect differences between males and females with SPS. We previously reported that female SPS rats did not show increased levels of N/OFQ in serum and CSF as the males did, and their anxiety-like symptoms were not evident at day 9 post-SPS (18), though allodynia and hyperalgesia were similar. However, SPS increased N/OFQ peptide in PAG and HYP of males and females at day 9, and only the N/OFQ increase in HYP was blocked by TNF-α antibody treatment. The 2-fold increase in ppN/OFQ mRNA in the PAG and prefrontal cortex in female rats after SPS is consistent with hyperalgesia and allodynia, even in the absence of increased N/OFQ in the serum. It was interesting that N/OFQ peptide levels in males increased in the HYP with SPS while NOP receptor mRNA also increased (**Table 2**). N/OFQ regulates the HPA axis primarily through its actions on the hypothalamus, so up-regulated disruption of peptide signaling would certainly be consistent with dysregulation of the N/OFQ-NOP system. Reversal of N/OFQ up-regulation in the hypothalamus by anti-TNF-α antibody treatment in males is consistent with alleviation of allodynia, hyperalgesia, and anxiety-like symptoms. Unlike what was reported for neuropathic pain, blockade of TNF-α action is therapeutic for female rats with traumatic stress-induced allodynia (89). However, in that study initiation of anti-TNF treatment was not begun until neuropathic pain was clearly established (1 wk after injury). Our study focused only on blocking TNF actions immediately after the trauma, before detection of allodynia. Pooley et al. (90) compared males and females in two different preclinical models of PTSD, including SPS. They found that in contrast to males, traumatic stress did not enhance negative feedback of the HPA axis in female rats. Clearly, much more work remains to understand the factors initiating traumatic stress and comorbid pain and

anxiety-like behaviors, and the differences between males and females at both initiation and maintenance phases of pain and anxiety-like behaviors.

In summary, this study suggests that TNF-α is integral in the pathophysiology of PTSD leading to the development of SPS-induced allodynia and hyperalgesia, and modulates changes in N/OFQ peptide and transcript. Acute treatment with a short-acting, small molecule TNF-α blocking drug biological may offer a novel therapeutic method to prevent or reduce symptoms of PTSD and co-morbid 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/s. Some of the data was previously presented in poster form.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center and the US Army Medical Research and Materiel Command Animal Care and Use Review Office.

AUTHOR CONTRIBUTIONS

PD and YZ performed animal experiments and biochemical and molecular studies. PD, YZ, and KS analyzed the data. KS, PD, RG, and MI designed the experiments. PD and KS wrote the manuscript. All authors discussed and commented on the manuscript.

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Association of Chronic Spontaneous Urticaria With Anxiety and Depression in Adolescents: A Mediation Analysis

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Huang Y, Xiao Y, Jing D, Li J, Zhang J, Chen X and Shen M (2021) Association of Chronic Spontaneous Urticaria With Anxiety and Depression in Adolescents: A Mediation Analysis. Front. Psychiatry 12:655802. doi: 10.3389/fpsyt.2021.655802

Background: Chronic spontaneous urticaria (CSU) is related to psychiatric comorbidities. It is not clear whether the relationship is affected by modifiable factors.

Objectives: To investigate whether the effect of CSU on anxiety and depression in adolescents is mediated by the symptoms of itching and sleep disturbance.

Methods: Questionnaire survey was conducted among newly enrolled college students. Dermatologists diagnose skin diseases, including CSU, during health examination. Anxiety and depression were measured by the Generalized Anxiety Disorder Scale and Patient Health Questionnaire, respectively. Sleep quality was measured by the Pittsburgh Sleep Quality Index. The symptoms of itching were measured by the numeric rating scale. According to the hypothesis, the mediating effect model was put forward and the structural equation model is used to build the mediation effect model. The mediation effect model was proposed according to the hypothesis and established using a structural equation model.

Results: A total of 2,358 students with no history of systemic disease and no pruritus disease (except CSU) were included in the analysis. A total of 393 CSU patients were included, and 1,965 healthy controls were selected based on age and sex matching. CSU was significantly associated with both anxiety and depression when the symptoms of itching and sleep quality were not modeled. A mediation model was proposed as CSU → itching → sleep disturbance → anxiety or depression. Itching and sleep quality mediated 65.4 and 77.6% of CSU's effects on anxiety and depression, respectively, and CSU had no significant direct effect on anxiety or depression in the mediation models.

Conclusions: The associations of CSU with anxiety and depression were mediated by the symptoms of itching and sleep disturbance. Effectively reducing the symptoms of itching thereby could increase natural sleep, which can further treat the emotional disorders among patients with CSU.

Keywords: chronic spontaneous urticaria, itching, sleep disturbance, mediation effect, depression, anxiety

INTRODUCTION

Chronic urticaria (CU) is a common skin disease characterized by the occurrence of wheals (hives), angioedema, or both lasting more than 6 weeks (1). CU can be classified into chronic spontaneous urticaria (CSU) and inducible urticaria (IU) (1–3). CSU occurs spontaneously with no obvious cause, while IU occurs when the formation of hives is reproducible after specific stimulus (4). CU affects 0.5–1% of the general population (5) and 0.1–0.3% of children (6). However, the prevalence of CU in adolescents is underappreciated. Globally, urticaria contributes to 4.7 million age-standardized disability-adjusted life years and 4.7 years lived with disability (7). Many studies have shown that patients with CSU often experience mental complications (8–10). The most common mental disorders observed in CSU patients are depression, anxiety, and somatoform disorders (8). The wide range of estimates of psychosocial factors among patients with CU was 16–96% (11). A recent meta-analysis found that CSU patients are six times more likely to suffer from anxiety and depression than healthy people (12). So, the psychiatric disorders associated with CSU should be taken seriously.

CSU is characterized by transient itching. Itching symptoms in patients with CSU have been described as stinging, tickling, and burning. Because of unpredictable attacks of pruritus and swelling, urticaria can seriously affect their quality of life (13). Itching is one of the main symptoms and the most important cause of sleep disturbance in patients with CSU. The severity of itching in CSU patients is associated with severe impairment of sleep quality (14–16). CSU severely affects sleep and quality of life, leading to difficulties related to work, family activities, social life, family relationships, sex, hobbies, and holidays.

Previous research has found that itching and sleep disturbance have a significant effect on mental disorders. Most clinical studies have revealed a correlation between mental disorders and chronic pruritus in patients with skin disorders. Chronic itching is associated with a high incidence of stress, anxiety, depression, and even suicidal thoughts, leading to major defects in quality of life (17–21). Sleep disturbance is significantly associated with increased risk of depression (22).

It is unknown whether CSU causes emotional problems through the symptoms of itching and sleep disturbance. Our study aims to investigate the mediation effect of the symptoms of itching and sleep disturbance on emotional problems in adolescents with CSU.

METHODS

Study Design

The College Student Skin Health Survey (CSSHS) (23) is a 4-year dynamic cohort of college students aiming to investigate skin health, diseases, and risk factors in adolescents. In the current study, we proposed a hypothesized pathway from CSU to emotional problems and tested the hypothesis using the baseline data of the cohort. Students from all over the country were admitted to four universities in four provinces (Hunan, Hubei, Fujian, and Xinjiang) and immediately performed health examinations and questionnaire surveys. All newly enrolled students who agreed to participate were classified as a cluster in September 2017 and September 2018. Participants that reported severe underlying diseases or the use of NSAIDs (24), or were diagnosed as other pruritic skin diseases, were excluded from the statistical analysis. We continuously selected patients with CSU from baseline data and matched healthy controls at a ratio of 1:5 based on age and sex matching.

Questionnaire and Measurements

During the physical examination, the University Student Affairs Department conducted a web-based questionnaire survey within 1 day. The questionnaire was self-reported (in computer rooms) and the questionnaire took 15 min to fulfill on average. The questionnaire was comprised of demographic information, history of diseases that might be associated with skin health, history of allergy, cigarette smoking, alcohol drinking, intake of soft drinks, water intake, food taste preference, defecation, sport, sleep quality, anxiety, depression, bath habit, skincare, and sun exposure. Anxiety and depression were measured by the two-item Generalized Anxiety Disorder Scale (GAD-2) and two-item Patient Health Questionnaire (PHQ-2), respectively. Sleep quality was measured by the Pittsburgh Sleep Quality Index (PSQI). The intensity of itching was measured by the numeric rating scale (NRS) of itching. For GAD-2, PHQ-2, PSQI, and NRS of the symptoms of itching, a higher score signified a greater level of anxiety, depression, sleep disturbance, and the symptoms of itching, respectively.

Clinical Evaluation and Diagnosis

Diagnosis of skin diseases and inquiry of disease history were performed by certified dermatologists during the health examination. Clinical manifestation, disease history, and family history of participants were inquired, and physical examinations were conducted to further diagnose CSU. During the health examination, qualified dermatologists conducted a skin disease diagnosis and medical history inquiry. The doctors asked the participants clinical manifestations, medical history, and family history and performed physical examinations to further diagnose CSU. CSU was diagnosed as wheals (hives), angioedema onset for 6 weeks or more with no obvious cause.

Statistical Analysis

Continuous data were presented as mean ± standard deviation, and between-group difference was tested using analysis of variance (ANOVA). Multiple comparisons were performed with the Least Significant Difference (LSD) *t*-test. Categorical data was presented as number (%), and the between-group difference was tested using the chi-square test.

The mediation effect model was performed to investigate whether CU affect emotional problems through itch and sleep disturbance. We proposed a model with a three-path mediated effect as: CSU (X) → itching (M1) → sleep disturbance (M2) → anxiety or depression (Y). As shown in **Figure 1A**, the total effect of predictor X on outcome Y is *c*; in **Figure 1B**, the direct effect of X on Y is *c'*, and the mediation effect of M1 and M2 is calculated as $a1 \times b2 + b1 \times a3 + a1 \times a2 \times a3$. The significance of the mediation effect was tested using the Bootstrap method.

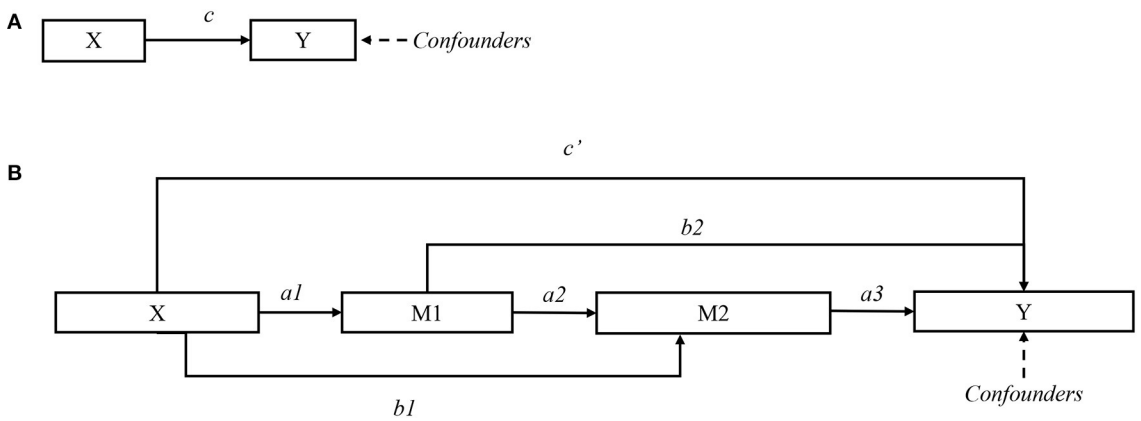

FIGURE 1 | Directed acyclic graph for mediation effect model. **(A)** Total effect *c*. **(B)** Direct effect *c'*; and mediation effects *a1* × *b2*, *b1* × *a3*, and *a1* × *a2* × *a3*.

TABLE 1 | Characteristics of participants, stratified by chronic spontaneous urticaria.

Characteristics	Total	Chronic spontaneous urticaria		*P*
		Case	Control	
Geographic region[a]				
North	246 (10.4)	38 (9.7)	208 (10.6)	0.198
Northeast	127 (5.4)	21 (5.3)	106 (5.4)	
East	573 (24.3)	74 (18.8)	499 (25.4)	
Central	664 (28.2)	121 (30.8)	543 (27.6)	
South	217 (9.2)	45 (11.5)	172 (8.8)	
Southwest	302 (12.8)	67 (17.0)	235 (12.0)	
Northwest	229 (9.7)	27 (6.9)	202 (10.3)	
Age (years)		18.3 ± 0.7	18.3 ± 0.7	1.0
Gender				
Male	1,128 (47.8)	188 (47.8)	940 (47.8)	1.0
Female	1,230 (52.2)	205 (52.2)	1,025 (52.2)	
Ethnicity				
Han	2,095 (89.0)	347 (89.0)	1,748 (89.0)	0.704
Other	263 (11.0)	46 (11.0)	217 (11.0)	
Annual household income (yuan)				
<10,000	185 (7.8)	18 (4.6)	167 (8.5)	0.001
10,000–29,999	470 (19.9)	63 (16.0)	407 (20.7)	
30,000–49,999	390 (16.5)	69 (17.6)	321 (16.3)	
50,000–99,999	571 (24.2)	100 (25.4)	471 (24.0)	
100,000–199,999	529 (22.4)	101 (25.7)	428 (21.8)	
≥200,000	213 (9.0)	42 (10.7)	171 (8.7)	

[a]*North: Beijing, Tianjin, Hebei, Shanxi, Inner Mongolia; Northeast: Liaoning, Jilin, Heilongjiang; East: Shanghai, Jiangsu, Zhejiang, Anhui, Fujian, Jiangxi, Shandong, Taiwan; Central: Henan, Hubei, Hunan; South: Guangdong, Guangxi, Hainan, Hong Kong, Macao; Southwest: Chongqing, Sichuan, Guizhou, Yunnan, Tibet; Northwest: Shaanxi, Gansu, Qinghai, Ningxia, Xinjiang.*

If the direct effect is insignificant and the mediation effect is significant, then mediators have a complete mediation effect on the outcome. $P < 0.05$ was considered statistically significant for all tests. Statistical analysis was performed in SPSS software 23.0 (IBM, NY, USA).

RESULTS

Based on screening of baseline data, we identified a total of 393 CSU patients, and a healthy control group of 1,965 was selected based on age and gender matching. The characteristics of the students stratified by CSU are shown in **Table 1**. Household income was associated with CSU.

In crude estimation, as shown in **Table 2**, students with CSU had significantly higher levels of the symptoms of itching, sleep disturbance, anxiety, and depression. Results remained consistent when dichotomizing the outcomes by certain cutoffs.

As shown in **Figure 2A**, CSU had significant total effects on sleep quality and emotional problems after adjustment for geographic region, age, gender, household income, food allergy, and drug allergy. One-mediator model is shown in **Figure 2B**: the direct effect of CSU on anxiety and depression was not significant; the symptoms of itching mediated 87.1 and 110.0% of CSU's effect on anxiety and depression, respectively (both $P < 0.001$). The two-mediator model is shown in **Figure 2C**: the direct effect of CSU on sleep quality, anxiety, and depression was not significant; the symptoms of itching and sleep disturbance mediated 95.7 and 116.7% of CSU's effect on anxiety and depression, respectively (both $P < 0.001$). The total effect, direct effect, and mediation effect are summarized in **Table 3**.

DISCUSSION

We proposed a mediation model from CSU to the symptoms of itching, sleep disturbance, and finally emotional problems in adolescents. The effects of CSU on anxiety and depression were fully mediated by the symptoms of itching and sleep disturbance, and CSU had no significant direct effect on anxiety or depression.

It is reported that at least 30% of patients with skin diseases have mental and psychosocial disorders (25–29). A systematic review found that the prevalence of depression in psoriasis

TABLE 2 | Comparison of itch, sleep quality, anxiety, and depression among adolescents with chronic spontaneous urticaria and those without.

Outcomes	CSU		Effect size		
	Yes	No	Difference (95% CI)	OR (95% CI)	*P*
Itch NRS	2.07 ± 2.13	1.02 ± 1.35	1.06 (0.90–1.22)		<0.001
Itch NRS ≥3	129 (32.8)	239 (12.2)		3.53 (2.75–4.53)	<0.001
PSQI	4.20 ± 3.14	3.54 ± 2.90	0.67 (0.35–0.99)		<0.001
PSQI ≥6	120 (30.5)	404 (20.6)		1.70 (1.31–2.16)	<0.001
GAD-2	0.99 ± 1.27	0.78 ± 1.10	0.21 (0.09–0.33)		0.001
GAD-2 ≥3	34 (8.7)	99 (5.0)		1.79 (1.19–2.68)	0.005
PHQ-2	0.97 ± 1.27	0.79 ± 1.14	0.18 (0.06–0.31)		0.005
PHQ-2 ≥3	34 (8.7)	109 (5.5)		1.61 (1.08–2.41)	0.020

CSU, chronic spontaneous urticaria; OR, odds ratio; CI, confidence interval; NRS, numeric rating scale; GAD, Generalized Anxiety Disorder; PHQ, Patient Health Questionnaire; PSQI, Pittsburgh sleep quality index; NRS, numeric rating scale.

patients ranged from 9 to 62%, and the prevalence of anxiety ranged from 11 to 43% (30). A recent study showed that depression, anxiety, and suicidal ideation are more common among patients with atopic dermatitis (31). The impact of CSU on quality of life was similar to that of cardiovascular disease (32). A systematic review showed that psychosocial factors had a prevalence of 46% in CSU patients (11). Our research is consistent with previous research results. The risk of anxiety and depression in urticaria patients is 1.79 times and 1.61 times that of normal people, respectively.

It is mentioned in "the European S2k Guideline on Chronic Pruritus" that all patients with urticaria have itching (33). It has also been reported that CSU is the source of itching in about 3% of children (34). A recent multicenter study found that compared with patients without itching, the depression and suicidal ideation of patients with skin diseases were strongly correlated with the presence of itching (35). This shows that the mental health problems of patients with skin diseases are largely related to itching. Other studies have confirmed this (36). One presumed reason for this correlation is that itching is associated with skin inflammation, which induces serotonin networks in the brain, leading to depression and anxiety (37, 38). Therefore, the symptoms of itching can be used as a major cause of mediating the occurrence of anxiety and depression in patients with CSU.

CSU has been reported to be associated with sleep disorders. Previous studies showed that patients with longer duration of CSU reported more sleep disturbance, tiredness, and irritability (39). Caine found that comparison of the Nottingham Health Profile scores shows that sleep disruption was a bigger problem for patients with CU. O'Donnell et al. reported that 38% of CU patients reported marked sleep disruption, and another 54% had sleep interference (40). A study in Singapore showed that many patients had difficulty falling asleep and often woke in the night (14). The CU-Q2oL scores from the German population showed that the greatest burdens of QoL were sleep, itching/embarrassment, and mental status (41).

Longitudinal studies have shown that subjective sleep disturbance is the main risk factor for the first-onset and recurrent depressive episodes of young and old people in the future (22, 42–44). Poor sleep quality can produce negative cognitions and emotions that are not conducive to sleep, such as anxiety and anger. As these vicious cycles continue, the risk of depression increases (45). One study showed that people with persistent insomnia have an average increased risk of depression by 3.7 times compared with those without insomnia (46). A meta-analysis found that people with insomnia have twice the risk of depression compared to people without sleep disorders (22). A systematic study of the relationship between adolescents' sleep duration and emotions found that sleep duration has a significant negative impact on the various emotional states of healthy adolescents (47). In a recent meta-analysis, it was found that lack of sleep may bring the risk of anxiety-related symptoms (48). Classic H1-antihistamines increase daytime sleepiness and reduce sleep quality scores, which have a negative impact on mood. The second-generation antihistamines have little effect on mood and are better than H1-antihistamines (49). Therefore, we believe that increasing sleep quality and natural sleep are more beneficial for anxiety and depression in patients with urticaria.

According to previous studies, the relationship between CSU and sleep disorders and the relationship between sleep disorders and anxiety and depression have been found. We can speculate that sleep disorder is a mediator of anxiety and depression in CSU patients.

In our study, when modeling itching as the only mediator, it mediated 87.1 and 110.0% of CSU's effect on anxiety and depression, respectively. By contrast, when modeling itching as the first and sleep quality as the second mediator, they mediated 95.7 and 116.7% of CSU's effect on anxiety and depression, respectively. The two-mediator model explained significantly greater variations than the one-mediator model. This is a further evidence of the importance of sleep disorders in CSU-associated emotional problems.

The study has limitations. First, the study was a case-control study, and the level of evidence limits the capability of causal relationship inference. Longitudinal observations are needed to confirm this proposed mediation model. Second, the study population was limited to highly homogeneous adolescents aged around 18 and having a similar educational background. The generalizability of the findings might be limited to other populations. Third, owing to the feasibility of study

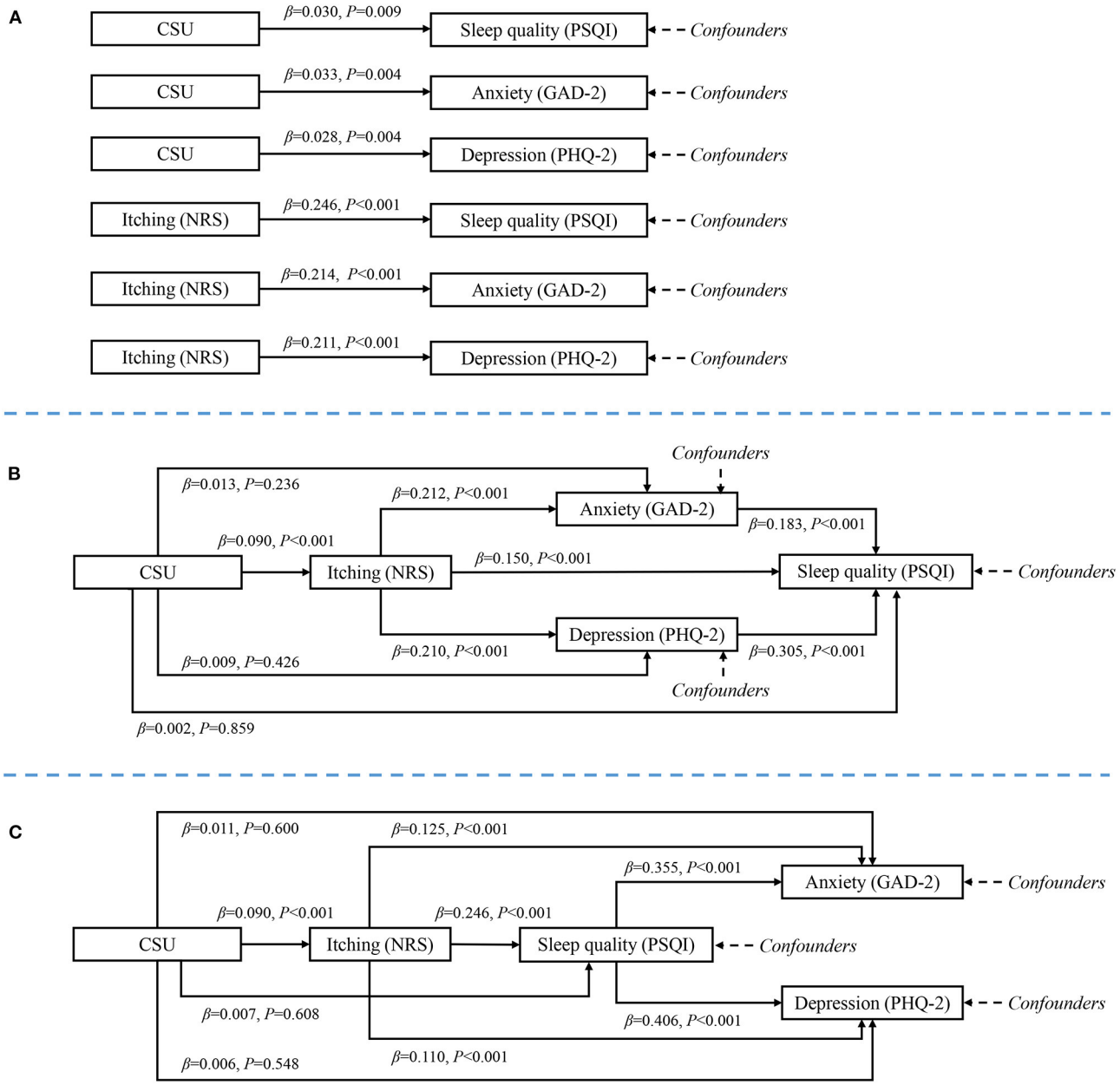

FIGURE 2 | Mediation model from chronic spontaneous urticaria to emotional problems. **(A)** Total effects, adjusted for confounders but not adjusted for mediators. **(B)** Direct and mediation effects of one-mediator model. **(C)** Direct and mediation effects of two-mediator model. Chronic spontaneous urticaria had significant total effect on sleep quality, anxiety and depression; but when mediators were modeled, urticaria had no significant direct effect on sleep quality, anxiety and depression. Itching and sleep quality had significant mediation effect.

implementation, anxiety and depression were only measured by two brief tools that were generally applied in clinical settings. Fourth, the urticaria activity score (UAS) scale and treatment status were not included in the CSU patients' inquiries, which may have a certain influence on the results.

The study also has strengths. First, this was a population-based epidemiologic survey among adolescents, and it had a higher level of evidence than the hospital-based studies. Second, the mediation model from CSU to the symptoms of itching, sleep disturbance, and emotional problems was clinically rational; the findings might provide potentially effective methods of an intervention targeting on the mediators. Third, the questionnaire survey and diagnosis for CSU were conducted using standardized methodologies.

In summary, we proposed a mediation effect model to test whether CSU was associated to anxiety and depression

TABLE 3 | Effect of predictors and mediators for sleep quality, anxiety, and depression.

			Total effect[a]		Direct effect[b]		Mediation effect[c]		
Predictor (X)	**Mediator (M)**	**Outcome (Y)**	**Size[d]**	***P***	**Size[d]**	***P***	**Size (95%CI)[d]**	***P***	**%**
Itching	Sleep quality	Anxiety	0.238	<0.001	0.123	<0.001	0.113 (0.098, 0.128)	<0.001	47.5
Itching	Sleep quality	Depression	0.252	<0.001	0.143	<0.001	0.110 (0.095, 0.125)	<0.001	43.7
Itching	Anxiety	Sleep quality	0.271	<0.001	0.168	<0.001	0.098 (0.055, 0.141)	<0.001	36.2
Itching	Depression	Sleep quality	0.271	<0.001	0.164	<0.001	0.103 (0.058, 0.148)	<0.001	38.0
CSU	Itching	Sleep quality	0.084	<0.001	0.015	0.458	0.069 (−0.067, 0.205)	<0.001	82.1
CSU	Itching	Anxiety	0.070	0.001	0.010	0.644	0.061 (0.005, 0.117)	<0.001	87.1
CSU	Itching	Depression	0.060	0.004	−0.005	0.818	0.066 (0.005, 0.0.127)	<0.001	110.0
CSU	Itching → sleep quality	Anxiety	0.070	0.001	0.003	0.870	0.067 (−0.008, 0.142)	<0.001	95.7
CSU	Itching → sleep quality	Depression	0.060	0.004	−0.011	0.558	0.070 (−0.007, 0.147)	<0.001	116.7
CSU	Itching → anxiety	Sleep quality	0.084	<0.001	0.011	0.546	0.073 (−0.116, 0.262)	<0.001	86.9
CSU	Itching → depression	Sleep quality	0.084	<0.001	0.017	0.359	0.068 (−0.115, 0.251)	<0.001	81.0

CSU, chronic spontaneous urticaria.

[a] *Total effect refers to the effect of predictor (X) on outcome (Y), adjusted from confounders but not mediators (M).*

[b] *Direct effect refers to the effect of predictor (X) on outcome (Y), adjusted for confounders and mediators (M).*

[c] *Mediation effect refers to the product of standardized coefficients of predictor to mediator and mediator to outcome, and the significance is tested by Bootstrap method. Percentage of mediation effect is calculated as: mediation effect/total effect ×100%.*

[d] *Expressed as standardized regression coefficient.*

through mediators including the symptoms of itching and sleep disturbance. We combined the known evidences from literatures and validated the hypothesis in adolescents. Our research suggests that effectively reducing the symptoms of itching thereby could increase natural sleep, which can reduce emotional problems among CSU patients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This study was conducted according to the guidelines laid down in the Declaration of Helsinki. All procedures involving patients were approved by the institutional research ethics boards of Xiangya Hospital, Central South University (Changsha, China). Informed consent was obtained from all students before the investigation.

AUTHOR CONTRIBUTIONS

YH, YX, and DJ performed the dermatological examinations. Senior dermatologists JZ and JL were responsible for quality control for diagnoses. MS and YH analyzed the data and drafted the manuscript. MS and YX designed the questionnaire. MS, JL, JZ, and XC designed the study and critically reviewed and revised the manuscript. MS and XC obtained the funding. All authors participated in the field survey and data collection and gave final approval to the version submitted for publication.

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The Aftermath: Post-pandemic Psychiatric Implications of the COVID-19 Pandemic, a South Korean Perspective

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The coronavirus disease 2019 (COVID-19) pandemic has disrupted our everyday life. Along with the fear of getting infected or of having loved ones infected, the lifestyle changes and the socioeconomic consequences of the pandemic have profound impact on mental health of the general population. While numerous studies on immediate psychological responses to COVID-19 are being published, there is a lack of discussion on its possible long-term sequelae. In this study, we systematically reviewed and meta-analyzed longitudinal studies that examined mental health of the general population prior to and during the pandemic. Furthermore, we explored the long-term psychiatric implications of the pandemic with data from South Korea. Our analysis showed that the number of suicidal deaths during the pandemic was lower than the previous years in many countries, which is in contrast with the increased depression, anxiety, and psychological distress in the general population in South Korea as well as in other countries. To explain this phenomenon, we propose a possibility of delayed impacts. The post-traumatic stress, long-term consequences of social restrictions, and maladaptive response to the "new normal" are discussed in the paper. COVID-19 being an unprecedented global crisis, more research and international collaboration are needed to understand, to treat, and to prevent its long-term effects on our mental health.

Keywords: COVID-19, post-pandemic, mental health, general populations, honeymoon phase, new normal, South Korea

INTRODUCTION

The coronavirus disease 2019 (COVID-19) evolved into a worldwide pandemic infecting more than 220 million individuals and claiming 4.5 million lives worldwide as of September 9, 2021 (1). The pandemic has brought considerable disruption to the way most people live, work, study, and access health care. These changes and their socioeconomic consequences, along with the fear of getting infected or of having loved ones infected have a profound impact on the mental health of the general population. Previous research about past pandemics, such as the 2003 outbreak of Severe Acute Respiratory Syndrome (SARS), has shown higher rates of depression, anxiety, insomnia, and post-traumatic stress in the general population (2, 3) as well as in people with pre-existing mental illness, health care workers, and survivors of severe cases of the disease (4, 5). As one of the first

countries to be affected by COVID-19 (6), South Korea's early implementation of testing, contact tracing, and social distancing has been recognized worldwide as successful measures that brought the virus under control (7). However, despite the well-recognized efforts, the country has recently faced at fourth wave of the viral epidemic with its peaks reaching over 2,000 newly confirmed cases per day, far above previous outbreaks.

There is a growing number of reports about mental health impacts of the COVID-19 outbreak (8–10), as well as the physical health consequences of COVID-19, in many countries such as China (11–15), Italy (16, 17), India (18, 19), Mexico (20), the United Kingdom (21, 22), the USA (23), and Spain (24, 25). In marked contrast to the rapidly growing literature "during" the COVID-19 pandemic, there is lack of published discussion on the mental health of the general population "after" the pandemic (26–28). Here, we aim to raise public awareness of putative prolonged impacts of the COVID-19 pandemic on mental health through a review of current knowledge on the impact of the pandemic on the mental health of the general population, and of data from South Korea (29, 30). The hypothesis of the present study is that COVID-19 has detrimental effects on mental health and suicide rates. We furthermore discuss the maladaptive response to a "new normal" lifestyle triggered by the COVID-19 pandemic.

METHODS

Study Selection and Data Extraction

A systematic search was conducted for longitudinal studies that measured changes in mental health of the general population since the COVID-19 pandemic that were published from January 1, 2020 to July 12, 2021. Electronic searches using subject headings (i.e., MeSH terms) and free-text keywords (an example shown in **Supplementary Table 1**) involved five electronic databases: PubMed, Scopus, Web of Science, APA PsychInfo, and CINAHL. According to the indices of each database, key search terms used for mental health included "mental health," "mental illness," "mental disorder," "depression," "anxiety," "stress," "post-traumatic stress disorder," and "suicide." Key search terms used for COVID-19 included "coronavirus disease 2019," "novel coronavirus," and "SARS-CoV-2."

Authors independently screened the titles and abstracts, and reviewed the full text articles to select studies meeting the following criteria: studies (a) with longitudinal designs; (b) that assessed psychological symptoms before and during the COVID-19 pandemic using the same measurement tools; (c) that are validated and standardized. The study selection process is shown in **Supplementary Figure 1** and the characteristics of included studies are shown in **Supplementary Table 2**. For more details about meta-analysis, see **Supplementary Materials**.

RESULTS

Three hundred sixty-one citations were retrieved from the electronic databases and 10 citations were identified through manual search. Three hundred thirty-one studies remained after removing duplicates and 165 after screening titles and abstracts. We assessed full-text articles and selected 22 articles for systematic review. Among the 22 articles, seven studies were excluded from the quantitative synthesis as their reported outcome values were not comparable to that of other studies; for instance, Ramiz et al. was excluded from the final meta-analysis as it only reported the prevalence of anxiety with a cut-off GAD-7 > 4 (mild or greater anxiety) (31), while most of the studies reported prevalence of anxiety of clinically significant level, equivalent of GAD-7 $\geq$ 10. All included studies were repeated cross-sectional studies, while five of them were conducted in the USA, four in the UK, two in the Netherlands, one in Japan, and others in other European countries.

Depression

Ten studies quantified and compared the level of depression in the general population before vs. during the pandemic. The studies measured the level of depressive symptoms using scales – such as the Patient Health Questionnaire (PHQ-9, PHQ-8, or PHQ-2), the Depression, Anxiety and Stress Scale (DASS-21), or the Brief Symptom Inventory (BSI) – or estimated the prevalence of major depressive episode using the Mini International Neuropsychiatric Interview (M.I.N.I.). The overall pooled odds ratio was 1.97 (95% CI: 1.26–3.09, see **Figure 1A**), showing a significant increase in depression since the pandemic. A high degree of heterogeneity was found across the studies ($I^2 = 97.6\%$, Q = 319.3, $p < 0.001$). We performed a sensitivity analysis to explore the impact of measurement tool by limiting the analysis to the studies using the PHQ and found consistent results: the overall pooled odds ratio was 2.75 (95% CI: 1.26–6.04, see **Supplementary Figure 2A**). (32, 33) reported no change or decrease in the prevalence of high Anxiety and Depression Symptoms (ADS) levels in the Dutch population-based longitudinal studies, which were measured using the 5-item Mental Health Index (MHI-5). The two studies did not report any measure specific to depression, thus were excluded from the meta-analysis. In an exploratory analysis with a cut-off PHQ-9/-8 $\geq$ 5 (mild or greater depression), there was no significant change in the prevalence after the pandemic (**Supplementary Figure 2B**).

Anxiety

Six studies were included in the meta-analysis for the change in the prevalence of clinically significant level of anxiety before vs. during the pandemic, defined as GAD-7 $\geq$ 10 or GAD-2 $\geq$ 3. The overall pooled odds ratio was 2.04 (95% CI: 1.08–3.83, see **Figure 1B**), suggesting a significant increase since the pandemic. A high degree of heterogeneity was found ($I^2 = 97.7\%$, Q = 317.6, $p < 0.001$).

Psychological Distress/Stress

Ten studies compared the prevalence of clinically significant level of psychological distress, defined as the 12-item General Health Questionnaire (GHQ-12) $\geq$ 4 or the 6-item Kessler Psychological Distress Scale (K6) $\geq$ 13. The overall pooled odds ratio was 1.62 (95% CI: 1.15–2.30, see **Figure 1C**). The heterogeneity among studies was high ($I^2 = 99.2\%$, Q = 648.0, $p < 0.001$).

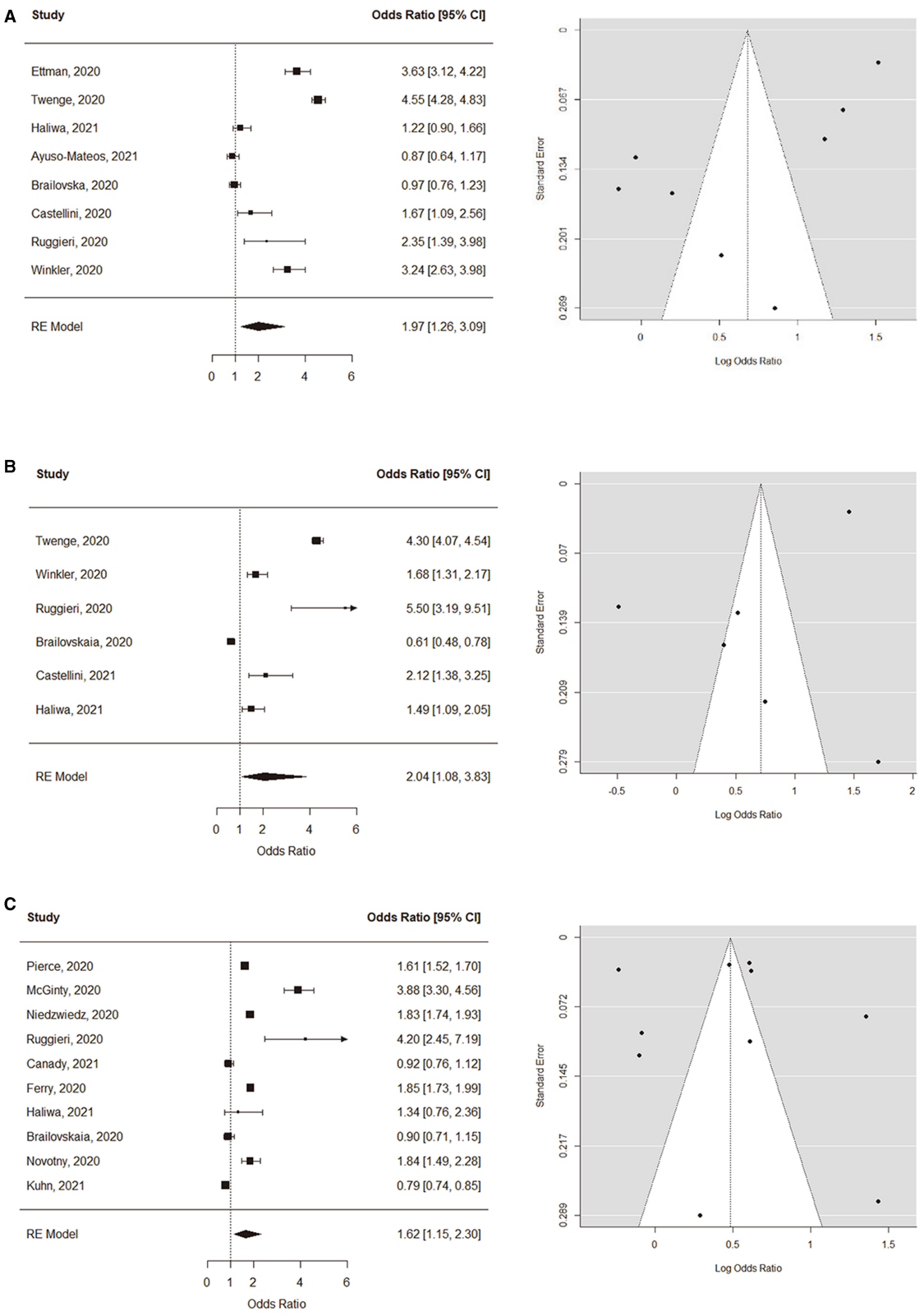

FIGURE 1 | Forest and funnel plots of symptom level comparisons. **(A)** Depression, **(B)** Anxiety, and **(C)** Psychological distress and stress.

Publication Bias Assessment

Funnel plots were first created for visual inspection to determine whether the included studies showed publication bias (see **Figure 1**). Egger's regression tests were additionally performed, and no significant publication bias was found in the studies regarding depression ($\beta = 1.22$, CI: 0.29–2.16, $p = 0.20$), anxiety ($\beta = 10.46$, CI: −1.08 to 2.00, $p = 0.71$), or psychological distress ($\beta = 0.33$, CI: −0.22 to 0.88, $p = 0.47$).

DISCUSSION

To our knowledge, this is the first systematic review and meta-analysis of longitudinal studies that examined the change in prevalence of psychiatric symptoms in the general population since the COVID-19 pandemic. According to our analysis, there was a significant increase in depression (OR = 1.97), anxiety (OR = 2.04), and psychological distress (OR = 1.62) in the general population. Since the beginning of the COVID-19 pandemic, experts warned of its possible destructive impact on the mental health of the general population. Although substantial heterogeneity exists between the studies on mental health during the pandemic, we can conclude that the virus outbreak has led to significant consequences in mental health in the affected populations.

Suicidal Ideation and Suicide Rates

A number of psychiatrists pointed out the possibility of an increase in suicide rates (34, 35), as the rise in psychiatric symptoms may remain untreated and be accompanied by socioeconomic burden. In contrast to such concerns, and to the results of our meta-analysis that showed increased depression, anxiety, and psychological distress, the suicidal ideation and suicide rates have not shown a remarkable rise since the pandemic.

A comprehensive review on the suicide behaviors during the pandemic reported no increase during the pandemic above pre-pandemic levels (36, 37). A couple of more recent studies, which investigated the impact of the COVID-19 pandemic on suicidal ideation in the general population though a longitudinal survey, in which measurements occurred in both pre-pandemic and pandemic periods, also showed no change or a small decrease (38, 39). Similarly, Google searching for suicide-related queries in Italy, Spain, the USA, the UK, and worldwide significantly declined after the pandemic declaration (40–42), although they increased again since the announcement of lockdown in each country (41).

A recent study that collected and analyzed real-time suicide data from 21 countries concluded that the actual number of suicides in the context of the COVID-19 pandemic in many countries remained unchanged or declined in the early phase of the pandemic compared with the anticipated levels based on the pre-pandemic period (43). The case of Japan, another country to among one of the first to be affected by the pandemic, is interesting. During the initial 5 months following the first wave of the outbreak (February to June 2020), the number of suicides decreased by 14% as in other countries, while after the second wave (July to October 2020), suicide rates increased, notably in greater magnitude among women, children, and adolescents (44, 45).

National Survey on the Mental Health in South Korea

The results of the most recent national survey on the mental health of South Korean population, fourth since March 2020, have recently been announced by the Korean Society for Traumatic Stress Studies (KSTSS) (29). The surveys reported that the level of anxiety – i.e., population at risk of clinically significant level of anxiety, defined as a General Anxiety Disorder-7 (GAD-7) score > 10 – increased in reaction to the outbreak and decreased when the virus seemed to be contained. However, the level of depression – i.e., population at risk of clinically significant level of depression, defined as a Patient Health Questionnaire-9 (PHQ-9) score > 10 – continued to increase from 17.5 to 20.0% from March 2020 to January 2021. This is a remarkable escalation from the rate of 3.8% reported by the Community Health Survey done in 2018 (29). The number of people with suicidal idea also increased from 9.7 to 13.4% from March 2020 to January 2021 (29). This suggests that while people suffer with anxiety in reaction to the severity of the outbreaks, they continue to accumulate symptoms of depression as the epidemic continues. Female and younger age (19–29 years old) groups were associated with a greater risk of depression (29). These findings are consistent with some systematic reviews on the subject (46–48).

In contrast, the number of suicides seems to decrease in Korea (49). Using the monthly provisional number of suicides reported by Korean Statistical Information Service (30), we performed a chi-squared test to evaluate differences in the monthly distribution of suicides during the COVID-19 pandemic compared with the previous year (2019 vs. 2020). A highly significant difference in distribution of deaths by suicide ($\chi^2 = 36.20$, df = 11, $p = 0.0002$) was shown, which were found in both genders (Male: $\chi^2 = 21.00$, df = 11, $p = 0.0335$; Female: $\chi^2 = 47.74$, df = 11, $p = 0.00002$). Overall, the total number of suicides from January to December 2020 is 781 cases lower than that of the same period in the previous year. However, while males showed decreases in the number of suicides throughout the year, females showed slight increases in number in March, April, June, August, and September (**Figure 2**).

Delayed Impacts of the COVID-19 - Honeymoon Phase

How can we explain the mismatch between the psychological response and the change in number of suicides? In the initial phase after a natural disaster, literature suggests there may be a brief decrease in suicide rates, a phase called the "honeymoon phase" (50, 51). There is much energy, optimism, and altruism that creates community bonding, accompanied by readily available assistance. This phenomenon has been observed in many national disasters including Hurricane Katrina in 2005 (52), the 9/11 terrorist attack in 2001 (53), the Great East Japan Earthquake in 2011(54), and the outbreak of Severe acute respiratory syndrome (SARS) in Hong Kong (55). However, as

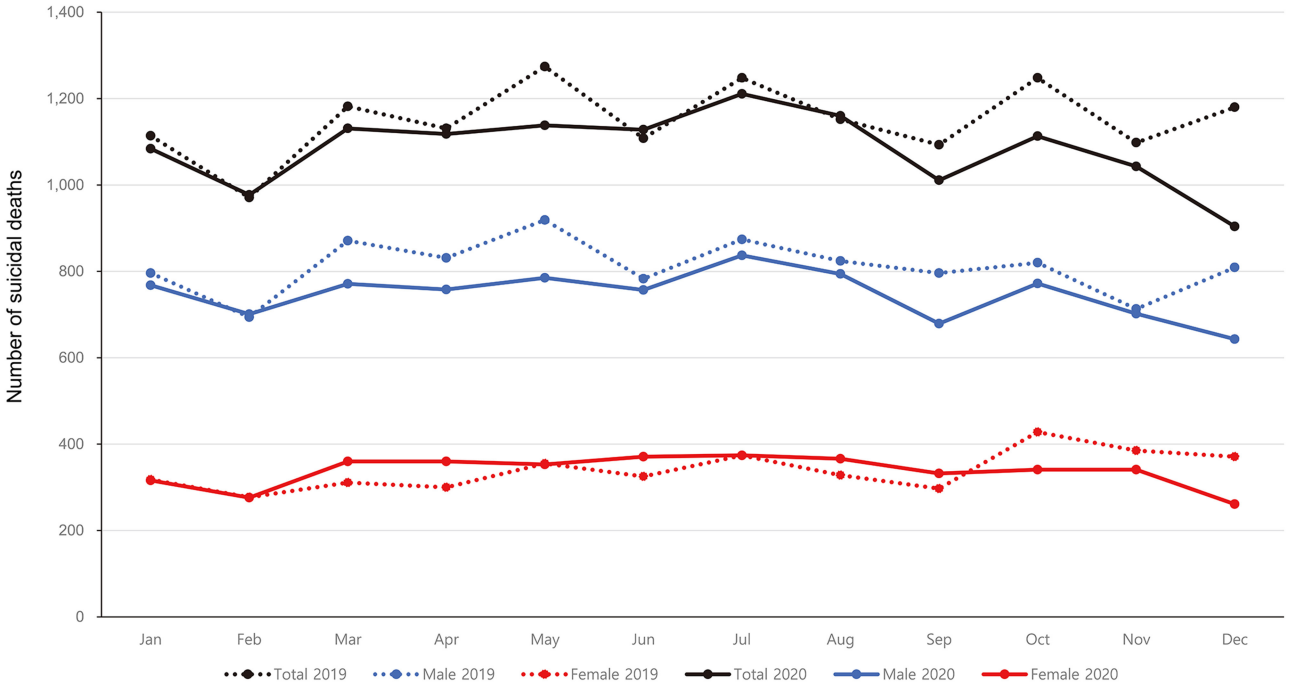

FIGURE 2 | Number of suicidal deaths in South Korea (provisional values).

time goes on, the honeymoon phase comes to an end. The socioeconomic and psychological burdens remain, while the assistance may discontinue. In Japan, about 80% of the cash benefits (an amount of approximately $940) was distributed to all citizens before June and claims for business subsidies grew rapidly in the beginning of the epidemic, which may have contributed to the initial decline in suicide rates (45). Whereas, a downtrend has been observed in the suicide rates of many countries in the early phase of the pandemic, one cannot readily assume the upcoming picture to be optimistic.

Long-Term Psychiatric Sequelae of the COVID-19

As coronavirus vaccines are being rolled out in many countries, the aftermath of the pandemic and what it represents for our mental health must be considered. While the infected cases are commonly accompanied by insomnia, anxiety, impaired cognitive function during the acute stage, they can extend to symptoms of post-traumatic stress disorder (PTSD), anxiety, and depression in the post-illness stage of coronavirus infection (56). Longer-term psychiatric sequelae remain unknown. The people closest to the infected cases—the families and frontline healthcare workers—have also experienced acute and post-traumatic stress (57). In the larger scope, no one could escape the stress and fear of getting infected or of having their loved ones infected. This generalized fear of illness and uncertainties contributed to the elevated anxiety and depression among the general population in many countries. How will this affect the future of mental health?

Social isolation, quarantine measures, and consequent deregulated emotions led to an increase in parental stress, children's psychological problems (58), and family violence (59, 60). Notably, adverse childhood events, such as childhood maltreatment, contribute to the development of psychiatric disorders, interfering with a child's brain development (61). Convincing evidence also suggests that pregnant women were more likely to experience anxiety and depression, and were more vulnerable to domestic violence during the pandemic (62, 63), while maternal mental illnesses have well-known adverse consequences for infant and child development (64). During the Spanish flu in 1918, the birth cohorts *in utero* displayed reduced educational attainment, increased rates of physical disability, lower income, and lower socioeconomic status in the USA (65). Similarly, children and adolescents today are also affected by the extreme social distancing measures such as school closures and lockdown restrictions. Besides the consequences on their immediate mental health (66), social restrictions can hamper the development of their social brain. Wearing masks also limits the children's experience of learning to read facial expressions, and the subtle nuances in language and communication (67). All this suggests the psychiatric sequelae of the COVID-19 can last for decades to come.

Mental Health in the "New Normal"

The pandemic crisis brought an acceleration of the Fourth Industrial Revolution, transforming every facet of our society. While new technologies such as teleconferencing and the Internet of Things permeate our everyday lives nowadays, many remain unable to adapt to such rapid digitalization of

social infrastructure, causing them anxiety ("techno-anxiety") or aversion thereto – "techno-phobia." At the opposite end, the extensive and compulsive use of internet and smartphones can cause "techno-addiction." A systematic review by La Torre et al. suggests that information overload and its constant availability can cause a condition called "techno-stress," characterized by higher circulating levels of cortisol, poor concentration, memory disturbances and irritability (68).

The COVID-19 pandemic has also created the need to implement and expand the use of remote communication technologies in health care. While traditional face-to-face interventions are disrupted and group therapies discontinued due to social distancing, telepsychiatry opens new opportunities for patients, allowing them to easily and safely access their mental health services (69, 70). Use of different technology platforms and settings such as applications can also be helpful for efficient monitoring and delivery of appropriate interventions (71). Although more evidence is required to examine the cost-effectiveness of digital psychiatry compared to the "in-person" care, the increase in demand for digital psychiatry is likely to improve its availability and quality in the market.

CONCLUSION

To conclude, although the COVID-19 pandemic will be contained 1 day, its psychiatric impact can persist both as direct and indirect consequences of the viral infection and as responses to the "new normal." Its effects can be generalized in every aspect of our society, manifesting themselves among adults, children, families, and in workplaces. Over the past century, globalization allowed infectious diseases to rapidly spread around the world. Severe Acute Respiratory Syndrome (SARS-CoV) and Middle East Respiratory Syndrome (MERS-CoV) were recent examples, but the COVID-19 pandemic is incomparable in terms of its extensiveness in time and place. As infectious epidemics can recur and their psychiatric implications will follow, it is crucial to expand our funding and international collaboration to understand the impact of epidemics on mental health.

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 authors.

AUTHOR CONTRIBUTIONS

SM, JWK, and YA contributed to the literature review, data acquisition, analysis, and interpretation. The first draft of the manuscript was written by SM. YHJ, JK, JWK, and YA read and revised the manuscript and approved the final version. All authors conceptualized 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/fpsyt.2021.671722/full#supplementary-material

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