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Plasma neuropeptides as circulating biomarkers of multifactorial schizophrenia

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ABSTRACT

Background: Promising biomarkers would be used to improve the determination of diagnosis and severity, as well as the prediction of symptomatic and functional outcomes of schizophrenia.
Basic procedures: In this study, we used three different mouse models induced by a genetic factor (PV-Cre; ErbB4$^{-/-}$, G group), an environmental stressor (adolescent social isolation, G group), and a combination of genetic factor and environmental stressor (PV-Cre; ErbB4$^{-/-}$ mice with isolation, G × E group). Attenuated PPI (%) confirmed the successful establishment of three schizophrenia-like mouse models. To evaluate whether neuropeptide levels in plasma would be potential biomarkers of different schizophrenia models in our work, we used MILLIPLEX® MAP method to simultaneously measure 6 critical neuropeptides in plasma.
Main findings: Among the evaluated neuropeptides, increased neurotensin tends to be associated with genetic factors of schizophrenia, increased orexin A seems to be a biomarker of an interplay between genetic and social isolation, while higher plasma oxytocin might be more apt to be responsive to social isolation. The potential biomarkers are mostly independent of sex.
Conclusions: This research would provide novel clues to develop circulating biomarkers of plasma neuropeptides for multifactorial schizophrenia.

1. Introduction

Schizophrenia is a severe and chronic mental disorder with a lifetime prevalence of approximately 1% worldwide. Although some subtle behavioral or cognitive deficits might be detected in an earlier developmental stage, psychotic symptoms emerge likely until late adolescence or early adulthood. Schizophrenia has been discovered to be a complex neurodevelopmental disorder, which is affected by multiple genetic and environmental factors, with the heritability about 80% [1–3]. However, the progress in the understanding of underlying etiology and identifying potential diagnostic or predictive biomarkers for schizophrenia has not been successful in general.

Neuregulin 1 (NRG1) and it's receptor ErbB4 have been reported as susceptibility genes of schizophrenia [4–6], and their expression appeared to be altered in the brains of patients with schizophrenia [7]. Wen et al. found that NRG1 played a critical role in balancing brain activity with PV-positive neurons as a target of NRG1/ErbB4 signaling in schizophrenia-like behaviors [8]. NRG1/ErbB4 mouse models for schizophrenia have been widely used due to their genetic predisposition to schizophrenia. Studies investigating schizophrenia-like phenotypes and underlying mechanisms demonstrated that PV-Cre; ErbB4$^{-/-}$ (PV-Cre; ErbB4$^{loxP/loxP}$) mice could serve as a schizophrenia model [8].

Socially isolated mice maintained in individual cages during brain maturation were studied to mimic social isolation in humans [9]. Behavioral alterations as a result of social isolation during adolescence have translational relevance to the core symptoms present in schizophrenia [10–12]. For example, deficits in prepulse inhibition (PPI) of acoustic startle [13] were similar to those seen in schizophrenia [14]. Social isolation paradigms in adolescents also model the impact of environmental insults on brain development and irreversible changes in neurochemistry at the cellular and molecular levels [15].

As no single mouse paradigm completely reproduces the diverse schizophrenia symptoms, “dual-hit” mouse models have been used to generate more comprehensive deficits, especially cognitive and

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negative features [10,16,17]. A "dual-hit" model, considering both genetic and environmental influences, was a boon to investigate the etiology of schizophrenia [17]. In this study, we exposed the established transgenic mouse model with a PV-Cre dominant-negative ErbB4 to a three-week adolescent isolation stress to investigate whether different models induce individual schizophrenia-like changes. Toward this end, the development of promising biomarkers, especially those in peripheral blood, is of clinical importance and practical significance for early detection and diagnosis of schizophrenia [18,19].

Neuropeptides are synthesized in the cell body and undergo anterograde transport to the nerve terminal. They are widely distributed throughout the brain and are often co-localized and co-released with monoamine neurotransmitters such as dopamine, glutamate, or γ-aminobutyric acid (GABA) [20]. It has been suggested that alterations in neuropeptide signaling lead to symptoms of schizophrenia, and consequently, the reversal of dysregulated neuropeptide signaling represents a possible novel treatment for the disorder [21,22]. At present, the majority of pharmacological treatments for schizophrenia are ligands that interact with the monoaminergic transmission. By co-localizing with the classical monoaminergic transmission, neuropeptides present reciprocal neurotransmitter interactions or independent activity in preclinical animal models [20,23,24].

Identifying reliable circulating biomarkers is an important approach to facilitate monitoring, therefore overcoming the limitation of infeasible brain tissue. In this study, we studied four groups of mice: wild-type mice without isolation (Control, C group); PV-Cre; ErbB4$^{-/-}$ without isolation (genetic factor, G group); wild-type mice with 3-week adolescent isolation (environmental stressor, E group); and PV-Cre; ErbB4$^{-/-}$ with 3-week adolescent isolation (combination of genetic and environmental factors, which was designated as G × E group). We evaluated the levels of α-MSH, β-endorphin, neurotensin, orexin A, oxytocin, and substance P in plasma, all of which are released into the circulatory system. In this study, we tried to explore whether the evaluated neuropeptides were promising biomarkers in different schizophrenia models represented by G, E, or G × E groups.

2. Materials and methods

2.1. Animals

As described previously [25], PV-Cre; ErbB4$^{-/-}$ mice (G group) were generated by a LoxP/Cre strategy, which crossed PV-Cre mice with LoxP-flanked ErbB4 mice. The PV-Cre; ErbB4$^{+/+}$ mice served as the control group [8]. Mice were genotyped using an established PCR protocol with the following LoxP primers: Sense, 5′-AAATCATCCTCTTGTGTGCTTTTGTAC-3′; Antisense, 5′-CTCGGTACTGCTGTTCCAGGTCAGA-3′; and the following PV-Cre primers: Sense, 5′-AAATCATCCTCTTGTGTGCTTTTGTAC-3′; Antisense, 5′-CTCGGTACTGCTGTTCCAGGTCAGA-3′.

Both males and females were included in the study. The experimental schedule is outlined in Fig. 1. The mice were divided into four groups: wild-type mice without isolation (control, C group); PV-Cre; ErbB4$^{-/-}$ without isolation (genetic factor, G group); wild-type mice with 3-week isolation (environmental stressor, E group) [17]; and PV-Cre; ErbB4$^{-/-}$ with 3-week isolation (combination of genetic factor and environmental stressor, which was designated as G × E group). There were 7–10 mice per group. All the mice were housed in a controlled environment (23 ± 1 °C; 12-h light/dark cycle) with access to water and food ad libitum. This study was carried out in accordance with the principles of the Basel Declaration and recommendations of the International Association of Veterinary Editors. The protocol was approved by the Institutional Animal Care and Use Committee of West China Hospital.

2.2. Prepulse inhibition (PPI) test

The mice were placed in a startle apparatus (Med-Associates, US), and whole-body startle movements were converted to digital signals using a computer. A continuous 70 dB background noise was present throughout the test. The test began with a five minute acclimation to the apparatus. The PPI test session consisted of three testing blocks. Block 1 (Habituation 1) included 10 sequential 120-dB trials, in which mice were acclimated to the 120-dB startle pulse-alone intensities. Block 2 (prepulse intensities) included 40 pseudo-randomized startle trials consisting of one of the following five conditions [17]: (a) no stimulus used to measure baseline movement in the startle chamber; (b) a 40-ms, 120-dB noise burst presented alone; (c–e) 100 ms pre-pulse (i.e., 74, 78, or 86 dB) followed by a 40-ms, 120-dB noise burst. Block3 (Habituation 2) included 10 sequential 120-dB trials which tested the overall habituation of the mice to the 120-dB startle pulse-alone intensities. Prepulse inhibition (PPI) of a startle response was calculated as % PPI = [(mean startle amplitude of pulse-alone trials − startle amplitude of prepulse-pulse trial)/mean startle amplitude of pulse-alone trials × 100], which indicated the percentage decrease in the amplitude of startle reactivity [26].

2.3. Plasma neuropeptide evaluation

Plasma was collected with EDTA as an anticoagulant. After collection, blood was mixed and centrifuged for 10 min at 1000*g*. Removed

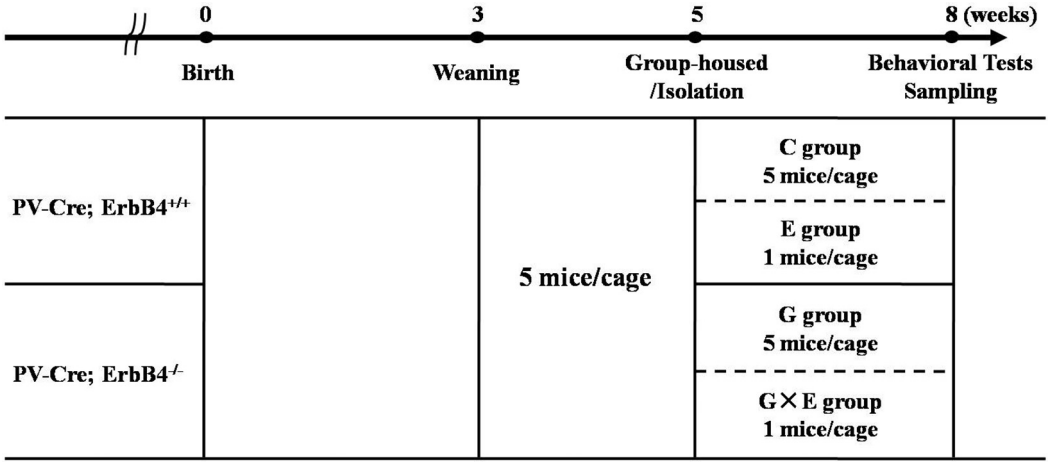

Fig. 1. Time schedule and graphical summary of groups. C group: PV-Cre; ErbB4$^{+/+}$ mice, 5 mice/group from 5 weeks to 8 weeks; G group: PV-Cre; ErbB4$^{-/-}$ mice, 5 mice/group from 5 weeks to 8 weeks; E group: PV-Cre; ErbB4$^{+/+}$ mice, 1 mice/group from 5 weeks to 8 weeks; G × E group: PV-Cre; ErbB4$^{-/-}$ mice, 1 mice/group from 5 weeks to 8 weeks.

plasma was stored at −80 °C. Plasma neuropeptides were evaluated using the Luminex® xMAP®-based MILLIPLEX® MAP Rat/Mouse Neuropeptide Magnetic Bead Panel (Cat. # RMNPMAG-83K). The neuropeptides include α-MSH, β-endorphin, neurotensin, orexin A, oxytocin and substance P. First, we added 375 μL of acetonitrile to a 250 μL sample in a microfuge tube. After incubation, the mixture was vortexed and centrifuged at 17,000*g* for 5 min. Then, 500 μL of supernatant was removed and dried using Speed Vac at the highest vacuum setting. Before assay, the dried samples were reconstituted with 200 μL Assay Buffer. The immunoassay was performed according to the protocol with a 50 μL prepared sample. Standard curves were generated using neuropeptide standards. Ran plate on FLEXMAP 3D® (Luminex, US) with xPONENT® software. The Median Fluorescence Intensity (MFI) data were analyzed using a weighted 5-parameter logistic method for calculating neuropeptide concentrations in samples.

2.4. Statistic analysis

In order to estimate the sample size, we used software G Power (http://www.gpower.hhu.de/) [27]. The parameters are set as follows: F tests as test family, one-way ANOVA as statistical test, $\alpha = 0.05$, power = 0.2, and number of groups = 4. Since reliable biomarkers require significant differences among groups, we set effect size f = 0.6. The estimated total number of mice needed in this experiment was 36. In this study, we used 7–10 mice in each group. The results of behavioral tests were analyzed using one-way ANOVA followed by the Dunnett post-hoc test. Neuropeptides evaluation was analyzed using one-way ANOVA followed by the Tukey's post-hoc test. The difference was considered statistically significant when $p < 0.05$. All the analyses were conducted using the SPSS18.0 statistical software package (SPSS Inc., Chicago, IL, USA). A summary of statistical analyses is displayed in Tables 1 and 2.

3. Results

3.1. General conditions

No death occurred during the whole eight-weeks of the observation period. Before the behavioral tests, all the enrolled mice displayed glossy fur, regular energy, normal whisker number and body weight, and without any significant differences in motor coordination. The mice did not exhibit any running nose or eye secretion, oral or nasal dryness or edema. The mouse feces were normal in color and regular in form, without pus, mucus, or blood. None of the groups manifested major physical changes. No obvious pathological changes were detected at necropsy.

3.2. Prepulse inhibition

All the groups produced similar startle responses to 120-dB stimuli (data not shown). A significant genotype effect on PPI was observed. The G group showed the impaired magnitude of PPI (%) compared with the C group in both male (Fig. 2A) and female mice (Fig. 2B) at all 3 dB. The results were coincident with previously reported results [8]. As an environmental stress, adolescent social isolation induced PPI (%) reduction in the E group. The joint effect of the genetic factor and social isolation during adolescence did not aggravate PPI (%) reduction. The trend of the attenuated PPI (%) in all groups was preserved in both sexes.

Table 1
Summary of statistical analyses of PPI analysis.

	Gender	Mean PPI (%)				ANOVA *p* value	Dunnett multiple comparison test		
		C	G	E	G × E		G vs. C	E vs. C	G × E vs. C
p74	Male	0.556	0.384	0.286	0.365	0.021	0.049	0.007	0.009
	Female	0.632	0.273	0.310	0.376	0.000	<0.001	<0.001	0.001
p78	Male	0.660	0.434	0.402	0.429	0.006	0.009	0.004	0.005
	Female	0.696	0.351	0.496	0.536	0.002	<0.001	0.033	0.047
p86	Male	0.658	0.530	0.389	0.523	0.001	0.049	<0.001	0.010
	Female	0.682	0.444	0.507	0.529	0.024	0.010	0.044	0.045

3.3. Plasma neuropeptide levels in different schizophrenia models

3.3.1. Neurotensin

In the male groups, the plasma neurotensin level was elevated significantly in PV-Cre; ErbB4$^{-/-}$ mice, both in group-housed (G group, $p = 0.043$) and in isolated mice (G × E group, $p = 0.018$) (Fig. 3A). However, the plasma neurotensin level was not altered after social isolation in PV-Cre; ErbB4$^{+/+}$ mice (E group, $p = 0.979$). In females (Fig. 3B), placed slightly differently, all three schizophrenia-like groups showed a significant increase in plasma neurotensin compared with C group (G group, $p = 0.016$; E group, $p = 0.005$; G × E group, $p = 0.000$).

3.3.2. Orexin A

Analysis from overall ANOVA revealed a significant difference in plasma orexin A levels among the four groups investigated (Fig. 4) in both males and females. Compared with the C group, post-hoc testing showed that plasma orexin A level of G × E group was significantly elevated (male, $p = 0.045$; female, $p = 0.019$), but without significant differences between the other two groups and C group.

3.3.3. Oxytocin

Mice with both genotypes (ErbB4$^{-/-}$ and ErbB4$^{+/+}$) showed significantly higher plasma oxytocin levels after adolescent social isolation. The effect of adolescent social isolation on both E and G × E groups was equivalent among males (Fig. 5A, E group, $p = 0.020$; G × E group, $p = 0.011$) and females (Fig. 5B, E group, $p = 0.016$; G × E group, $p = 0.013$). Meanwhile, the G group showed normal plasma oxytocin levels compared with the C group.

3.3.4. α-MSH, β-endorphin, and substance P

One-way ANOVA revealed no significant changes in plasma levels of α-MSH (sFig. 1), β-endorphin (sFig. 2) or substance P (sFig. 3) in both male and female groups.

4. Discussion

In this study, we established schizophrenia-like mouse models by taking both genetic and environmental factors into account. Attenuated PPI (%) has been widely reported in schizophrenia patients [14,28], their unaffected first-degree relatives [29], and patients with a schizotypal personality disorder [30]. In clinical research, deficient PPI (%) correlated with positive and negative symptoms [31], and neurocognitive measures [32] of schizophrenia. Attenuated PPI (%) in schizophrenic patients was also proposed as a biomarker [33] or an endophenotype [34] to reflect the link between genetic and clinical expression. In schizophrenia-like mouse models, PPI (%) has been used as an effective index tool to study genetic models [35], gene-environment interactions [36,37], and even successful in predicting antipsychotic medications [38]. The expected sensory gating deficits (the attenuated PPI of the acoustic startle response, which has been widely used to evaluate schizophrenia-like phenotypes including distractibility, inattention, and cognitive deficits in rodents) were induced in both PV-Cre; ErbB$^{-/-}$ and adolescent social isolation mice [8,39]. However, we did not find a synergistic effect on PPI in the mice exposed to genetic (PV-Cre; ErbB$^{-/-}$) and negative environmental risk (adolescent social isolation). We simultaneously measured six neuropeptides, including neurotensin, orexin A, oxytocin, α-MSH, β-endorphin, and substance P in mice plasma. We found that firstly, the ErbB4 gene-related schizophrenia mouse models have significantly elevated plasma neurotensin levels

Table 2
Summary of statistical analyses of neuropeptides.

	Gender	Mean (pg/ml)				ANOVA *p* value	Dunnett multiple comparison test					
		C group	G group	E group	G × E group		G vs. C	E vs. C	G × E vs. C	E vs. G	G × E vs. G	G × E vs. E
Neurotensin	Male	62.834	85.341	63.136	89.679	0.027	0.039	>0.999	0.048	0.037	0.968	0.043
	Female	47.953	72.742	77.696	86.922	0.004	0.040	0.009	0.001	0.940	0.356	0.691
Orexin A	Male	298.415	357.447	269.858	396.163	0.048	0.575	0.943	0.045	0.252	0.752	0.055
	Female	270.740	295.820	273.322	382.698	0.029	0.946	>0.999	0.041	0.942	0.159	0.041
Oxytocin	Male	101.864	96.144	179.305	182.510	0.006	0.996	0.044	0.049	0.010	0.010	>0.999
	Female	56.283	71.316	168.228	168.506	0.016	0.986	0.030	0.049	0.043	0.047	>0.999

in both sexes, which indicated that the social isolation alone could be a negative environmental stress affecting plasma neurotensin levels in females. Secondly, the synergistic effects of adolescent social isolation combined with genetic factors may increase plasma orexin A level. Thirdly, the change of plasma oxytocin levels was affected mainly by adolescent social isolation rather than the genetic modification in schizophrenia-like models. Our results suggested that different neuropeptides may be responsive to specific probable pathogenic factors for schizophrenia, and combined accurate biomarkers are needed for multifactorial schizophrenia.

Neuropeptides are small protein-like molecules synthesized in the cell body and undergo anterograde transport to the nerve terminal. They are neuronal signaling molecules secreted by the central and peripheral nervous systems. They influence a broad spectrum of biological functions including the regulation of metabolism, immunity, and behavior. More importantly, neuropeptides are often co-localized and co-released with monoamine neurotransmitters. Reciprocal interactions between neuropeptides and monoamine neurotransmitters serve as possible avenues to explore the mechanisms underlying schizophrenia and support pharmacological development [20].

Neurotensin, a 13-amino-acid neuropeptide, was isolated from bovine hypothalamic extracts and originally characterized with vasodilatory properties [40]. It is frequently co-localized with dopamine in the synaptic terminals [23]. In the prefrontal cortex (PFC), stimulation of local D2 autoreceptors suppresses dopamine release while increasing neurotensin release [41]. In the forebrain, neurotensin influences dopamine release via co-localization in dopaminergic terminals [24]. Activation of neurotensin receptors triggers

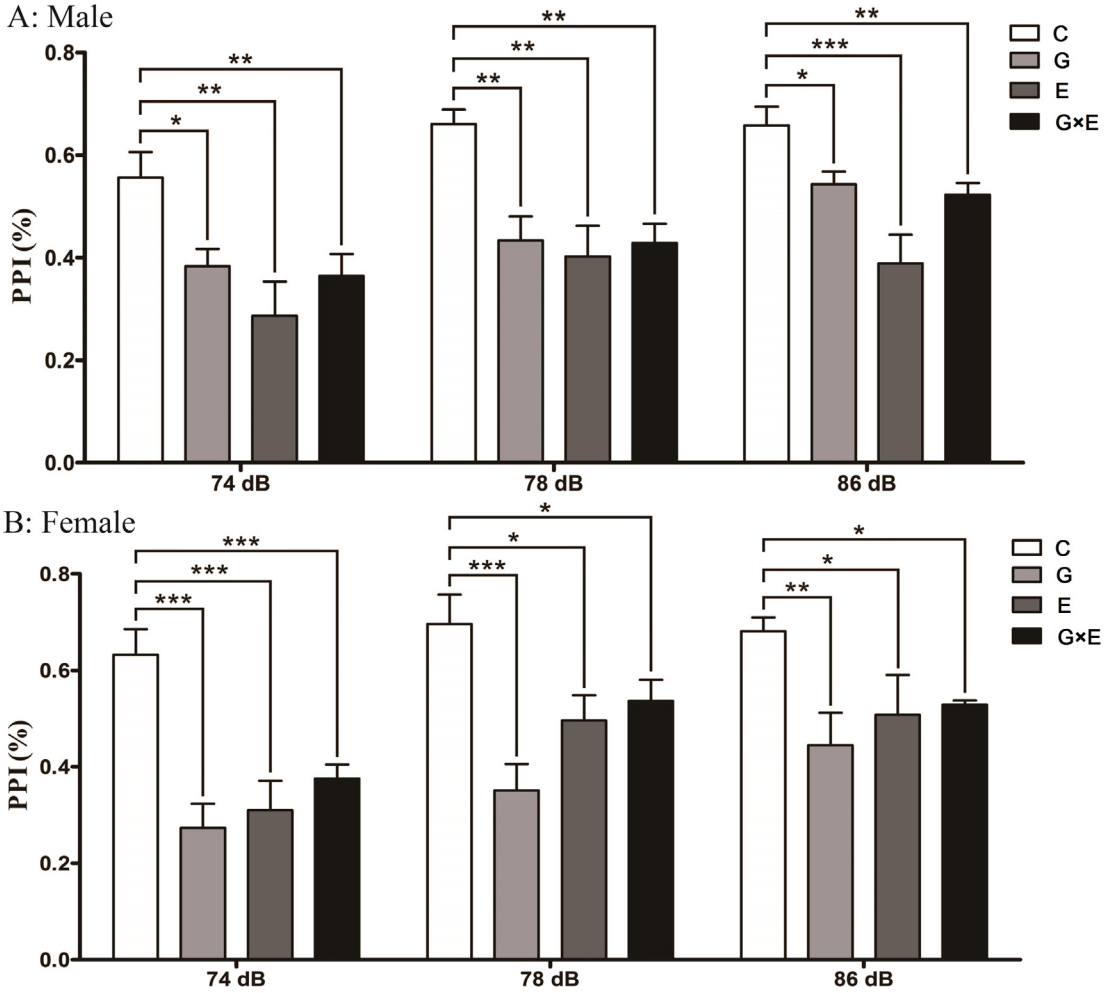

Fig. 2. Prepulse inhibition in different schizophrenia-like models. (A) All C group, G group, and G × E group displayed reduced PPI in male mice; (B) all C group, G group, and G × E group displayed reduced PPI in female mice.

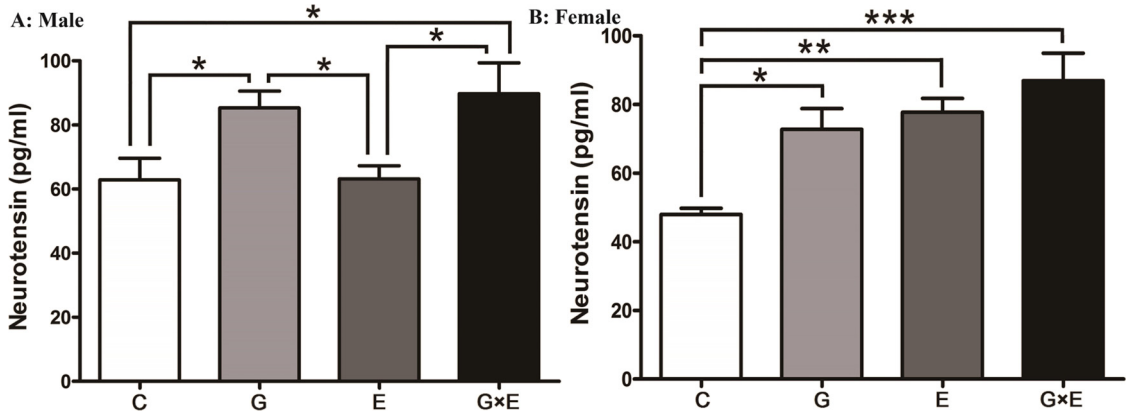

Fig. 3. Plasma neurotensin levels in different schizophrenia-like models. (A) Plasma neurotensin was increased in the G group and G × E group in male mice; (B) plasma neurotensin increased in C group, G group, and G × E group in female mice.

a spontaneous increase in the activity of mesocortical dopamine neurons in the ventral tegmental area (VTA) [42]. As reported, twenty-one drug-naive schizophrenic patients showed a bimodal distribution of neurotensin levels in cerebrospinal fluid (CSF). In a subgroup of 9 schizophrenic patients, their neurotensin levels were markedly lower than the rest of the patients and controls, while the neurotensin levels of 11 schizophrenic patients were comparable to those of healthy volunteers. Interestingly, there was a patient who had an extremely high neurotensin level, which is consistent with the ErbB4 gene-related schizophrenia mice model in our results [43]. This may suggest that NT has potential prospects as a genetic risk indicator for schizophrenia. Furthermore, neurotensin concentration was found to suffer from both genetic factors (Flinders sensitive line/Flinders resistant line) and environmental stress (Maternal separation affects) [44]. In the postmortem samples from 46 schizophrenic patients, neurotensin level in BA23 of frontal cortical was significantly elevated using radioimmunoassay [45]. Interestingly, there were shreds of evidence about the sex difference in neurotensin concentration after nicotine self-administration in rats [46] and as a response to acute antipsychotic drug administration [47]. According to the abnormal neurotensin levels, neurotensin or neurotensin receptors represent potential targets for the development of novel antipsychotic medications [48,49]. Unfortunately, in a Phase II double-blind clinical trial, SR48692 or meclinertant (a neurotensin antagonist) showed no efficacy in ameliorating positive or negative symptoms of schizophrenia [50]. A Phase I clinical trial of NT69L (a neurotensin analog with antipsychotic-like activity) was suspended for its potential hypotensive effect [51]. However, there is still a great deal of interest and excitement about the antipsychotic potential of neurotensin antagonists or analogs as novel treatments for schizophrenia.

Orexin A (33-amino-acid peptide) mediates feeding, stress, and activation of the sympathetic nervous system [52]. The cell bodies of orexin A-producing neurons are confined to the perifornical and lateral hypothalamic nuclei. Their axons project to midline-intralaminar thalamic nuclei predominantly, and to the prefrontal cortex and a few lower brain areas [53]. Complex neuroanatomical interactions exist between orexin A and dopaminergic systems in various regions of the brain, such as orexin-containing projections influencing dopamine neuronal activity and dopamine release in VTA [54] and nucleus accumbens (NAc) [55]. In this study, we did not find any changes in orexin A level in either social isolation or genetic factor groups, which was consistent with the results reported before [56]. However, an increased plasma orexin A level was detected in the schizophrenia-like models with synergistic effects of adolescent social isolation combined with genetic factors (Fig. 4), which was also found in a subgroup of schizophrenia patients associated with fewer negative and disorganized symptoms [57]. The combination of social isolation and genetic factors may have a correlation with the fewer negative and disorganized symptoms. Interestingly, we found that the G × E group of mice displayed a slightly increased body weight, although without statistical significance (data not shown). As a neuropeptide stimulating food intake, the significantly increased plasma orexin A level may be relevant to the slight

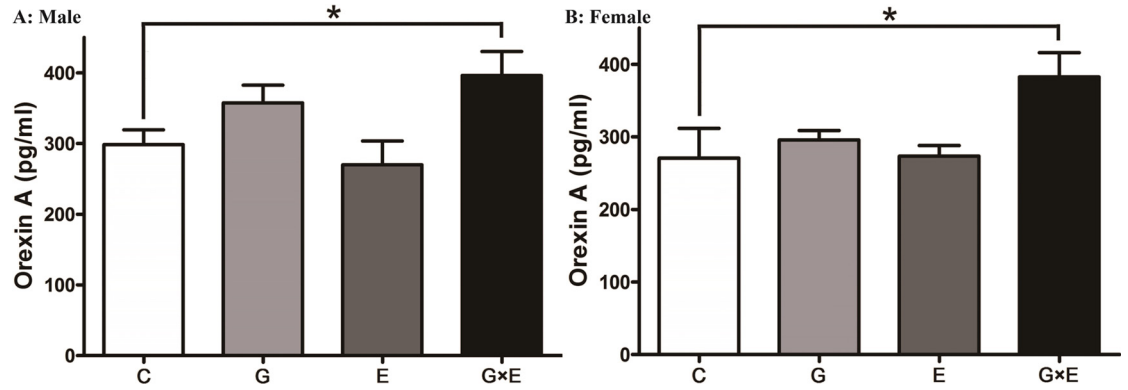

Fig. 4. Plasma orexin A levels in different schizophrenia-like models. (A) G × E group displayed increased plasma orexin A level in male mice; (B) G × E group displayed increased plasma orexin A level in female mice.

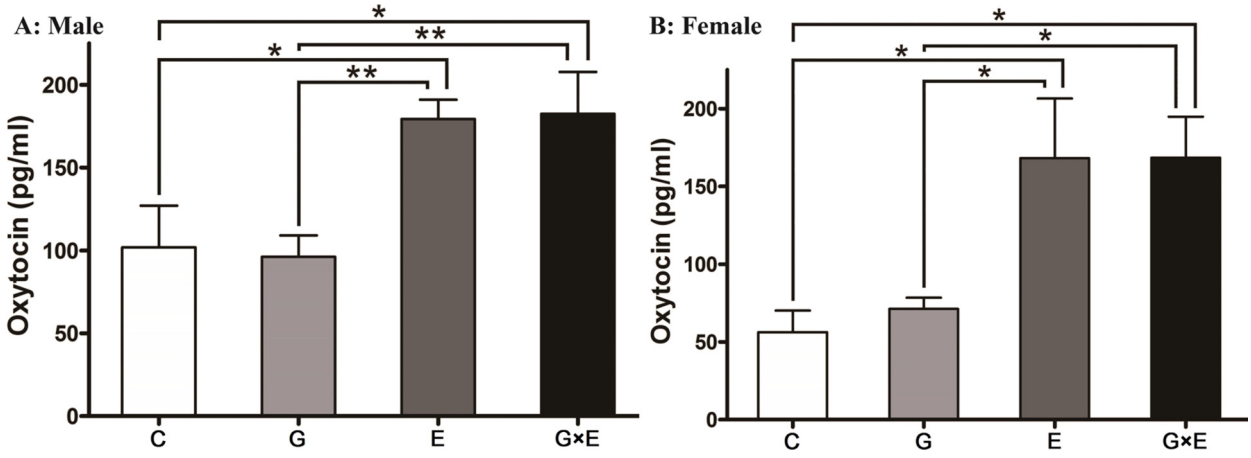

Fig. 5. Plasma oxytocin levels in different schizophrenia-like models. (A) Both E group and G × E group exhibited increased plasma oxytocin levels in male mice; (B) both E group and G × E group exhibited increased plasma oxytocin levels in female mice.

increase in body weight. The orexin activation of the dopamine system may be the basis of some of its mechanisms in psychiatry like schizophrenia and depression. New therapeutic strategies to either activate the orexin system in depression or inactivate it in schizophrenia may be effective approaches in the treatment of such disorders [58–60]. However, large multicenter clinical trials are needed to validate the findings.

In the brain, oxytocin is primarily found in magnocellular neurosecretory cells in the paraventricular and supraoptic nuclei of the hypothalamus. It is also expressed in the cortex, limbic system, and spinal cord. In the posterior pituitary, oxytocin is released directly into the bloodstream. Association analysis revealed that polymorphisms in oxytocin and oxytocin receptor genes are associated with the risk of schizophrenia as well as a response to antipsychotic treatment [61], which provided a window for developing antipsychotic drugs. The levels of oxytocin in schizophrenics appear to fluctuate; they increased [62,63], decreased [64], or were not significantly different [65]. In our research, we found an enhanced plasma oxytocin level after specific social isolation other than the genetic modification in schizophrenia-like models. It may be owing to up-regulated oxytocin-ir neurons in the paraventricular nucleus after isolation [66]. But in postmortem samples from schizophrenic patients study, both oxytocin receptor gene expression and binding sites in different brain regions were found significantly reduced [67], which might be a dose-offset effect of increased oxytocin concentration. Oxytocin levels also show mixed results following neuroleptic treatment [62,68]. However, the potential development of novel antipsychotic effects of oxytocin should not be ignored.

Several limitations in the present study should be acknowledged. Evaluation of the circulating neuropeptides in the plasma provides potential biomarkers for schizophrenia. However, it is not clear whether peripheral neuropeptide levels reflect their expression in the brain in this study. Therefore, caution is needed when trying to extrapolate the underlying mechanisms of schizophrenia. Furthermore, different biomarkers correspond to specific pathophysiological factors, so it is challenging to develop more convincing schizophrenia models to distinguish different biomarkers of multifactorial schizophrenia. To be noted, the combination of multiple neuropeptides may be one of the solutions to identify a specific biomarker for schizophrenia, which is worth further study in order to provide guidance for different pathogenesis (G, E, or GXE) of schizophrenia. Additionally, further investigations are necessary to explore the underlying mechanisms.

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Author contributions statement

PN, XT, XM, XH, and TL, designed the experiments.

PN, YT, XG, LY, JW, YW, LZ, YZ, CZ, and LL conducted experiments, collected data, and analyzed data.

PN and TL wrote the manuscript.

PN and TL supported this study financially.

Declaration of competing interest

We do not have anything to disclose.

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