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Decreased *Npas4* expression in patients with post-stroke depression

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**ABSTRACT**

**Purpose:** Post-stroke depression (PSD) is a frequent neuropsychiatric disorder following stroke which is associated with poor outcome. Neuronal Per-Arnt-Sim (PAS) domain protein 4 (*Npas4*) is associated with cognitive function. *Npas4* expression in peripheral blood mononuclear cells (PBMCs) from patients with PSD was measured to find new therapeutic strategy.

**Patients and methods:** Ischemic stroke patients (*n* = 152) within 1 week of stroke onset were recruited. At 3 months follow-up, the patients were divided into a PSD group (*n* = 77) and a stroke group (*n* = 75) using the Hamilton Rating Scale. Healthy subjects (*n* = 75) were also recruited in the study. The PSD group received 12 weeks of duloxetine treatment. Cognitive function was evaluated using the P300 test. *Npas4* expression in PBMCs was measured by quantitative RT-PCR (qPCR).

**Results:** Before treatment, P300 latencies in the PSD group were prolonged and the P300 amplitudes were lower than the control group (*P* < 0.01). *Npas4* expression in the PSD group was also lower than the control group (*P* < 0.01). After treatment, the P300 latencies were reduced and the amplitudes were significantly elevated in the PSD group compared to that before treatment (*P* < 0.01). Meanwhile, *Npas4* levels were significantly higher than that before treatment (*P* < 0.01). *Npas4* expression was positively correlated to the P300 amplitudes (*P* < 0.05).

**Conclusion:** Changes of *Npas4* expression in PBMCs are associated with cognitive impairment in PSD patients and new therapeutic options applying *Npas4*-related transcript mechanism could be considered in the future.

1 Introduction

Post-stroke depression (PSD) is a common neuropsychiatric disorder observed in stroke patients occurring in approximately one third of stroke survivors. It is associated with increased morbidity, mortality, and stroke-related complications, including significant social and cognitive impairment [1, 2]. Most of the patients with cognitive impairment after stroke cannot live independently and report a lower quality of life [3].

The basic helix-loop-helix-Per-Arnt-Sim (bHLH-PAS) family of proteins are dimeric transcription factors with a variety of distinct functions. Most bHLH-PAS proteins are associated with a variety of human diseases, including cancer, metabolic syndrome, and psychiatric conditions [4]. As a member of this family,
the neuronal PAS domain protein 4 (Npas4) is an early-response transcription factor which is enriched in the brain and induced in a subset of interneurons as well as excitatory neurons in neuronal activity dependent manner [5]. It participates in brain’s responses to environmental aggression, shaping behavioral and stress responses [6]. Studies have revealed that Npas4 plays a role in stroke through modulation of apoptotic and inflammatory pathways [7]. Differential levels of Npas4 expression in the brain may regulate anxiety, depression and cognition related disorders [8]. However, less is known about the role of Npas4 in the etiology of PSD.

Cognitive defects are common among PSD patients, but effective treatment for this condition is few. The aim of this study was to examine Npas4 level in the PBMCs of patients with PSD and find the correlation between Npas4 expression and cognitive impairment with PSD.

2 Methods

2.1 Study population

The study population consisted of 152 patients from the Second Affiliated Hospital of Xinxiang Medical University who were consecutively enrolled. Diagnostic criteria for enrollment were based on the stroke criteria from the Chinese 4th National Cerebrovascular Meeting in Chengdu, Sichuan Province, China, 10th ~ 13th Jan 1995. CT-scan and magnetic resonance imaging (MRI) were used for diagnoses. Stroke severity was evaluated by trained neurologists using the National Institute of Health Stroke Scale (NIHSS) at admission [9]. Depression was diagnosed by at least two experienced psychiatrists in accordance with the Diagnostic and Statistical Manual IV (DSM-IV) criteria [10]. None of the patients was treated with antidepressant drugs before recruitment. The severity of depressive symptoms was evaluated by the 24-item Hamilton Depression Scale (HAMD-24) [11]. Exclusion criteria included 1) co-morbidity that might affect outcome, such as cancer, dementia, or Parkinson disease; 2) history of depression or other psychiatric disorders before the incident stroke; 3) severe aphasia, dysarthria, or visual/auditory impairment; and 4) life expectancy of less than 3 months. For comparison, 75 control subjects with no personal or familial history of psychiatric illness were recruited. All subjects were free of severe physical diseases, including acute ischemic stroke.

Informed consent was obtained after proving verbal and written information to participants or nearest relatives when relevant. Ethics approval was granted by the Ethics Committee for Medical Research at the Second Affiliated Hospital of Xinxiang Medical University.

2.2 Groups and assessment

All subjects, including the 152 stroke patients and 75 control subjects, were classified according to the Chinese 4th National Cerebrovascular Meeting’s diagnostic criteria and the HAMD-24.

The stroke group (75 patients, 64.41 ± 8.13 years old) recruited first-ever stroke patients without depression and with HAMD-24 score < 8. The PSD group (77 patients, 63.00 ± 8.39 years old) was distinguished from the first-ever stroke patients with HAMD-24 score ≥ 21. The PSD group received duloxetine treatment according to a previous study [12].

The control group consisted of 75 individuals with a mean age of 65.64 ± 7.75. They were healthy without history of stroke, and their HAMD-24 score < 8.

2.3 P300 measurement

All subjects were evaluated after informed consent, the PSD group was tested again at 12 weeks after the treatment. The P300 measurement was taken in a shielded room with patients lying relaxed but alert. According to the international brain electrical 10/20 system, a reference electrode was put in the patient’s right ear lobe (A2), a ground on the forehead (FPz), and recording electrodes (Cz) were placed along the midline of the scalp. Electrode impedance was < 5 kΩ and P300s were recorded for 600 ms after stimulus started. Test (target) stimuli comprised 90 dB/4000 Hz tones (20% probability) and run between the 80 dB/1000 Hz nontarget stimuli (80% probability). Before the measurements, patients were given instruction and told what to expect. The patients were asked to focus in listening to the voice, and respond to the target tones by pressing the index finger of the superior hand. Before experiments were performed, subjects had to acknowledge they understood the study procedure. Patients were asked to press a button in response to the presentation of target stimuli and reaction time was recorded. The average response of two separate trials was recorded and analyzed.
2.4 Analysis of Npas4 expression

2.4.1 Sample preparation

Venous blood samples (5 mL) were collected from all subjects at the same time of P300 test. Peripheral blood mononuclear cells were obtained through centrifugation at 3000 rpm for 15 min and then stored at −80 °C for later analysis.

2.4.2 Quantitative RT-PCR

qRT-PCR was performed using a StepOnePlus Real-time PCR System (Applied Biosystems, USA) using TaqMan probes, according to the manufacturer’s instructions (Applied Biosystems, USA). Total RNA was extracted from peripheral blood mononuclear cells (200 μL) using RNAiso Plus (TaKaRa, Japan). cDNA was produced using M-MLV reverse transcriptase (Promega, USA). Primers and TaqMan probes for Npas4 were as follows: forward 5’-CTGCTTTGTAAATCATGGTA-3’, reverse 5’-ACAGGC-AGTAAATCCATG-3’, and TaqMan probe 5’-(FAM) TGTAGCCTCACCACCATCCTTG(Eclipse)-3’. Glyceraldehyde phosphate dehydrogenase gene (GAPDH) was used as the endogenous control gene: forward 5’-GGACCTGACCTGCCGTCTAG-3’, reverse 5’-TAGCCCAGG-ATGCCCTTGAG-3’, and TaqMan probe 5’-(FAM)CCTCCGACGCCTGCTTCACCT(Eclipse)-3’. The PCR samples were incubated at 95 °C for 30 s, followed by 45 cycles of 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 20 s. The mean Ct values of three replicates per sample were normalized to GAPDH to obtain corresponding ΔCt values. ΔΔCt values were calculated by subtracting the ΔCt of the control group from each ΔCt of the study groups. The relative change in Npas4 and expression was expressed by $2^{-ΔΔCt}$ [13].

2.5 Statistical analysis

Data was analyzed using SPSS, version 19.0 (SPSS Inc., Chicago, IL, USA). Quantitative data was expressed as a mean with standard deviation (SD). Data were analyzed using analysis of variance (ANOVA) with Dunnett’s test for multigroup comparisons and paired t-test was used to compare pre- with post-treatment changes. Statistical significance was defined as $P < 0.05$.

3 Results

3.1 Demographic data

The characteristics and demographics of the 227 subjects were listed in Table 1. All patients were examined in Hans-in, China. PSD group consisted of a greater percentage of females than other groups, who were more likely to live with offspring, and had higher widowhood percentage, stroke admission severity and white cell counts.

3.2 ERPs (P300)

As shown in Table 2, prior to treatment, the P300 latencies were significantly prolonged in patients with

| Table 1 | Baseline characteristics of patient groups. |
|---------|------------------------------------------|
|         | PSD (n = 77) | Stroke (n = 75) | Control (n = 75) | p* |
| Baseline characteristics | | | | |
| Age (years) | 63.00 ± 8.39 | 64.41 ± 8.13 | 65.64 ± 7.75 | 0.135 |
| Female population | 68.8% | 50.6% | 46.7% | 0.013 |
| Education (years) | 6.29 ± 3.41 | 6.09 ± 3.78 | 6.71 ± 2.80 | 0.520 |
| Widowhood | 27.3% | 11.5% | 13.1% | 0.009 |
| Admission median NIHSS score | 6.36 ± 1.91 | 4.64 ± 2.55 | 0 | 0 |
| Living with offspring | 50.6% | 72.1% | 67.2% | 0.018 |
| Laboratory findings | | | | |
| White cell count (10^9/L) | 7.15 ± 0.74 | 5.1 ± 0.86 | 5.43 ± 1.17 | 0.001 |
| Glucose level (mmol/L) | 4.94 ± 0.51 | 4.87 ± 0.37 | 4.86 ± 0.45 | 0.927 |
| TG level (mmol/L) | 2.53 ± 0.84 | 3.12 ± 1.14 | 2.70 ± 0.19 | 0.336 |
| Hs-CRP level (mg/L) | 1.41 ± 0.16 | 1.37 ± 0.62 | 1.19 ± 0.19 | 0.139 |

Results are expressed as either percentage or mean ± SD. TG, triglyceride; Hs-CRP, high-sensitivity C-reactive protein.
a, ANOVA with Dunnett’s test and the chi-square test were used.
PSD, stroke compared to controls ($P < 0.01$). The P300 amplitudes in patients after stroke were significantly reduced compared with the control group ($P < 0.01$). 12 weeks after treatment, the P300 latencies in PSD group significantly decreased (Fig. 1; $t = 15.08$, $P < 0.01$). Meanwhile, the P300 amplitude in PSD group significantly increased (Fig. 2; $t = −12.29$, $P < 0.01$).

### 3.3 Npas4 expression

Before and after 12 weeks of duloxetine treatment, $Npas4$ expression was determined by qRT-PCR. Data ($2^{-\Delta\Delta Ct}$) did not conform to the normal distribution ($P$ Shapiro-Wilk test $= 0.00$), therefore the data was transformed using the square root transformation $[\sqrt{2^{-\Delta\Delta Ct}}]$ to determine the relative change in $Npas4$ expression. Before treatment, the relative $Npas4$ level in the PSD group was significantly lower than that in the control group (Table 2; $P < 0.01$). While $Npas4$ expression in the stroke group was significantly higher than that in other groups (Table 2; $P < 0.01$). In addition, the $Npas4$ level in the PSD group increased significantly after 12 weeks of duloxetine treatment (Fig. 3; $t = −21.094$, $P < 0.01$).

### 3.4 Correlation analysis of P300 latency, P300 amplitude, and Npas4 expression in the PSD group

The latency of P300 was not found to be correlated with $Npas4$ expression in PBMCs of the PSD group (Fig. 4; $r = −0.135$, $P = 0.132$). There was positive association between P300 amplitudes and $Npas4$ expression in the PSD group (Fig. 5; $r = 0.197$, $P = 0.015$).

### Table 2 Comparisons of P300 and Npas4 expression before treatment.

| Group     | n   | P300 Latencies (ms) | P300 Amplitude ($\mu$V) | Relative Npas4 expression |
|-----------|-----|---------------------|-------------------------|---------------------------|
| PSD       | 77  | 368.42 ± 15.17*     | 3.39 ± 2.30*            | 0.81 ± 0.15*              |
| Stroke    | 75  | 346.39 ± 24.02*     | 5.53 ± 3.33*            | 1.72 ± 0.50*              |
| Control   | 75  | 323.60 ± 18.55      | 8.76 ± 2.46             | 1.09 ± 0.43               |
| $F$       |     | 99.75               | 74.31                   | 105.721                   |
| $P$       |     | 0.000               | 0.000                   | 0.000                     |

Results are expressed as mean ± SD,* $P < 0.01$, compared with control group.
Fig. 4 Correlation between relative Npase4 expression and P300 Latencies in patients with PSD.

Fig. 5 Correlation between relative Npase4 expression and P300 amplitudes in patients with PSD.

4 Discussion

About the pathogenesis of PSD has many hypothesis, suggesting that complex interactions among psychological, social, and biological factors are involved. Our study found that decreased Npas4 levels in PBMCs may be important in the pathophysiology of depression. One possible explanation is that patients with reduced Npas4 expression may be genetically vulnerable for depression. One study has demonstrated that adult Npas4 deficient mice showed behavioral deficits that resemble those observed in patients with mental disorders, including schizophrenia, social anxiety, despair, and so on [6, 8, 14]. Stress-induced reduction of Npas4 expression may precipitate in depressive disorder. Shimizu et al. proposed that stress could exacerbate depression through neuronal atrophy and death [15]. Decreased Npas4 level in PSD may fail to play a protective role in stress response and eventually contribute to the collapse of stress-adaption system.

The P300 provides information on conscious and unconscious cognitive functions as an event-related potential (ERP) [16]. The P300 amplitude indicates the subjects’ ability to receive outside information, and the P300 latency objectively reflects cognitive function, judgment, and other advanced thinking activities [17]. The majority of studies on P300 in cognitive impairment reported prolonged latencies and reduced amplitudes in visual or auditory modalities [18]. In this study, we found that patients after stroke have deficits in perception and/or cognitive processing. The cognitive impairment in stroke patients with depression was more serious than patients without depression, which can be restored by chronic antidepressant treatment. Furthermore, the level of Npas4 mRNA was reverted to well above the baseline value, thus we concluded that duloxetine treatment may ameliorate the cognitive impairment through the regulation of Npas4. It is important to point that Npas4 could bind to activity-dependent promoters I and IV in the Bdnf gene and enhance its expression level, which in turn control the number of γ-aminobutyric acid (GABA)-releasing synapses on excitatory neurons [14, 19]. Several studies have supported a central and causal role of the GABAergic system in the etiology of depressive disorders [20, 21]. Previous studies demonstrated that duloxetine was able to restore the physiological levels of Npas4 and GABAergic markers in SERT knockout rats. Based on these findings, a hypothesis was made that the ‘noradrenergic component’ of duloxetine may lead to the regulation of Bdnf transcripts and Npas4 [22].

A lot of limitations existed in the study. First, we were not able to assess risk factors such as nearly negative life accidents, family harmony, poverty and so on. Second, although we have acquired similar results in hippocampus of rats [23], few data supports Npas4 in PBMCs could represent its level in the brain, and we are not able to detect the level in human brain. Third, this study was conducted only in one hospital, but did not extend to other hospitals in China. Forth, Npas4 was only measured at study admission, so there is no data to reveal the dynamic changes in PSD patients before and after treatment.
In spite of these limitations, in this study, we demonstrated that Npas4 expression in PBMCs of PSD group significantly reduced, suggesting that Npas4 may play a role in the pathophysiology of PSD. In addition, decreased Npas4 expression was associated with cognitive impairment and its level was increased significantly after antidepressant treatment, suggesting the applicability of Npas4 related-transcriptional mechanisms may be an efficient and novel anti-depression target. Future studies examining the regulation of Npas4 will be required to enhance our understanding of the mechanisms of PSD.

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Disclosure

The authors declare that they have no possible conflict of interests including financial support, corporate involvement, patent holdings, etc.

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