AVP and Glu systems interact to regulate levels of anxiety in BALB/cJ mice

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Abstract: While the roles of glutamic acid (Glu), arginine vasopressin (AVP) and their respective receptors in anxiety have been thoroughly investigated, the effects of interactions among Glu, N-methyl-D-aspartic acid (NMDA) receptor, AVP and a-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor on anxiety are still unclear. In the present study, the agonist and antagonist of the NMDA receptor and AMPA receptor, as well as the antagonist of AVP V1 receptor (V1aR) were introduced into BALB/cJ mice by intracerebroventricular microinjection, and the anxiety-like behaviors of the mice were evaluated by open field and elevated plus-maze tests. Compared with C57BL/6 mice, BALB/cJ mice displayed higher levels of anxiety-like behavior. Significant anxiolytic effects were found in the NMDA receptor antagonist (MK-801) and the AMPA receptor or V1aR antagonist (SSRI49415), as well as combinations of AVP/MK-801 and SSRI49415/DNQX. These results indicated that anxiety-like behaviors expressed in BALB/CJ mice may be due to a coordination disorder among glutamate, NMDA receptor, AMPA receptor, AVP and V1aR, resulting in the up-regulation of the NMDA receptor and V1aR and down-regulation of the AMPA receptor. However, because the AMPA receptor can execute its anxiolytic function by suppressing AVP and V1aR, we cannot exclude the possibility of the NMDA receptor being activated by AVP acting on V1aR.

Keywords: Anxiety; AMPA receptor; NMDA receptor; AVP; V1aR

Many hypotheses have been proposed in regards to the pathogenesis of anxiety e.g. monoamine neurotransmitter receptor disorder, Glu system disorder, and neuronal plasticity. However, all these theories have limitations. In recent years, one of the most important targets in anxiety research has been the glutamic acid (Glu) system, which closely interacts with monoaminergic neurons, peptidergic neurons, and neurotrophic factors, and plays a vital role in the adjustment of emotion-directed behaviors (Hashimoto, 2011; Rios et al, 2009; Teuchner et al, 2010). Molecular and behavioral studies have shown that the N-methyl-D-aspartic acid (NMDA) receptor is critical in the neuronal plasticity of the prefrontal cortex (Chen et al, 2008; Fontán-Lozano et al, 2007; Valenzuela-Harrington et al, 2007). Furthermore, the antagonist of the NMDA receptor, MK-801, significantly down-regulates anxiety-like behaviors in animals (Herman & Cullinan, 1997; Müller & Holsboer, 2006), whereas, excessive activation of the NMDA receptor induces anxiety or depression (Hashimoto, 2011; Skolnick, 1999). However, the AMPA receptor agonist of Glu not only produces remarkable anxiolytic and anti-depression effects (McEwen, 1999), but also increases mRNA expression of brain derived neurotrophic factor (BDNF) and significantly reduces levels of anxiety and depression (Katz, 1982; Sapolsky, 2000).

Skolnick (1999) found that expression levels of arginine vasopressin (AVP) and AVP V1 receptor (V1aR) were higher in patients with anxiety than those in normal people. Appenrodt & Schwarzberg (2000) found that V1aR knock-out reduced levels of anxiety in mice, suggesting that excessive activation of V1aR greatly

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influenced levels of anxiety-like behavior in mice.

Correlations between the Glu and AVP systems have been indicated in many studies. Patchev et al (1994) found that the NMDA receptor and its agonist induced AVP release *in vitro*; however, intracerebroventricular microinjection of NMDA receptor antagonists, other than its metabotropic receptor (Yamaguchi & Watanabe, 2005), interdicted AVP release *in vivo* (Iwanaga et al, 2011; Yamaguchi et al, 2005). Intracerebroventricular microinjection of NMDA was found to increase levels of serum AVP (Maione et al, 1992), with AVP known to then increase excitatory responses of Glu and NMDA in the brain (Joëls & Urban, 1984). However, the mechanisms underlying the regulation of anxiety-like behaviors via the interaction between different types of Glu receptors and the AVP system are yet to be elucidated.

Animals' anxiety-like behaviors are determined by multiple factors with various mechanisms. Under anxiety, remarkable changes can be found in the levels of AMPA and NMDA receptors of Glu, as well as in V1aR expression. However, effects of their interactions on levels of anxiety are still not known. In the present study, we performed intracerebroventricular microinjection of drugs in BALB/cJ (BALB) mice, which are characterrized with innate high levels of anxiety-like behaviors, and then evaluated changes in levels of anxiety by open field and elevated plus-maze tests. We investigated the interactions and individual roles of Glu, AVP and their respective receptors in adjusting anxiety-like behaviors in adult BALB/cJ mice.

MATERIALS AND METHODS

Animals and grouping

Healthy four-week-old male BALB/cJ and C57BL/6 (C57) mice, weight 23±3 g, were provided by the Laboratory Animal Center of Xi′an Jiao Tong University, Xi′an, China. Animals were housed in the breeding room of the College of Life Sciences, Shaanxi Normal University, Xi'an, China, using wooden chips as bedding materials. The study animals were fed *ad libitum* with standard mice food. The light cycle was 12 D:12 L (08:00−20:00) and the temperature was set at 24±2 °C. Animals were allowed one week to accommodate. Due to the low sociality and high anxiety in BALB/cJ mice (Skolnick, 1999), C57 mice, which are characterized with high sociality and low anxiety, were taken as the normal controls. Animals were randomly placed into ten groups (eight animals in each group): (1) BALB/SAL group (BALB/cJ normal control group), animals were intracerebroventricularly microinjected with saline water; (2) C57/SAL group (C57 normal control group), animals were intracerebroventricularly microinjected with saline water; (3) BALB/AVP group, BALB/cJ mice were intracerebroventricularly microinjected with AVP; (4) BALB/AVP group, BALB/cJ mice were intracerebroventricularly microinjected with AMPA; (5) BALB/NMDA group, BALB/cJ mice were intracerebroventricularly microinjected with NMDA; (6) BALB/MK-801 group, BALB/cJ mice were intracerebroventricularly microinjected with NMDA receptor antagonist, MK-801; (7) BALB/DNQX group, BALB/CJ mice were intracerebroventricularly microinjected with AMPA receptor antagonist, DNQX; (8) BALB/AVP+MK-801 group, BALB/CJ mice were intracerebroventricularly microinjected with AVP and MK-801; (9) BALB/SSRI49415 group, BALB/CJ mice were intracerebroventricularly microinjected with V1aR antagonist, SSRI49415; (10) BALB/SSRI49415+DNQX group, BALB/CJ mice were intracerebroventricularly microinjected with SSRI49415 and DNQX.

Experimental reagents

AMPA, DNQX (AMPA receptor antagonist), NMDA, MK-801 (NMDA receptor antagonist) (Xu, 1999), AVP and SSRI49415 (V1aR antagonist) (Yayou et al, 2008) were obtained from Sigma, USA. Pentobarbital sodium was purchased from Merck, USA (repackaged by Chinese Pharmaceutical (Group) Shanghai Chemical Reagent Company).

Stereotactic surgery

Animals were anesthetized with 2% pentobarbital sodium (40 mg/kg, i.p), with the head fixed on a WDT-II stereotaxic instrument. Accurate coordination of the lateral ventricle (AP: –0.46 mm, RL: ±1.2 mm, H: –2.25 mm) was determined by referring to the stereotactic atlas of mice (Paxinos & Franklin, 2004). A stainless steel catheter (internal diameter=0.47 mm) was stereotaxically implanted into the lateral ventricle and was then secured to the skull using phosphate cement and dental acrylic. Animals were postoperatively injected with penicillin (10^5 U) for 3 days, and were allowed to eat and drink *ad libitum*. One week's recovery was allowed before the commencement of the intracere-

broventricular microinjections (Maeng et al, 2008; Yayou et al, 2008).

Intracerebroventricular microinjection

Reagents were prepared with saline water, including AMPA (0.5 μg/μL) (MacMaster et al, 2008), NMDA (0.5 μg/μL) (McKinnon et al, 2009), DNQX (1.68 μg/μL), MK-80 (0.5 μg/μL) (MacMaster et al, 2008), AVP (0.5 μg/μL) and SSRI49415 (0.5 μg/μL) (Yayou et al, 2008). Target reagents (0.1 μL) were infused into bilateral ventricles smoothly by a micromanipulator (CMA402, Swiss) through the planted catheter (Müller & Holsboer, 2006). Each microinjection was completed within 1 min and the micromanipulator was kept in the position for another minute to prevent effusing. An interval of 10 min was necessary when reagents were given in combination.

Animals were euthanized immediately after behavioral tests. Their brains were collected, fixed and sectioned (40 μm). Samples with inaccurate coordination (less than 10% in each group in this study) were excluded from statistical analysis.

Evaluation of anxiety-like behaviors

Open field test

Experimental mice were placed into an open field test arena (50 cm×50 cm), the bottom of which was divided into 16 squares (12.5 cm×12.5 cm). The 16 squares were further divided into two areas, with the surrounding 12 squares considered as area 1 and the four squares in the middle considered as area 2. Individual mice were videotaped with a camera mounted above the center of the open field. Animals were allowed to stay in the arena for 5 min, individually, and the percentage of time spent in the central area, frequency of total transitions, and total moving distance were recorded. The arena was deodorized with 5% alcohol after each trial. Animal behaviors were analyzed via the versatile VideoMot2 video tracking system (TSE, Germany). The anxiety level of the animals decreased with increasing time spent in the central area (area 2). High frequency of total transitions indicated that the animal was more explorative. Long moving distance represented high locomotor activity.

Elevated plus-maze

The elevated plus-maze (50 cm high) consisted of two open and two enclosed arms, 30 cm×5 cm, interconnected by a 5 cm square central area. Individual mice were placed into the central area and were under observation for 5 min. Time spent in the open arms, frequency of transitions to each arm and total moving distance were recorded. The maze was deodorized with 5% alcohol after each trial. Animal behaviors were analyzed via VideoMot2. The anxiety level of the animal decreased with increasing time spent in the open arms. High frequency of arm intercross indicated the animal was more explorative. Long moving distance represented high locomotor activity.

Statistical analysis

All behavioral variables were analyzed via SPSS-10.0 (SPSS inc., Chicago, IL, USA). Data were expressed as mean±*SE*. Differences between groups were determined by One-way ANOVA, with $P<0.05$ considered statistically different and $P<0.01$ considered significantly statistically different.

RESULTS

Open field test

ANOVA results showed that drug treatments significantly influenced time spent in the central area ($F_{(9, 87)}$= 18.22, P=0.0013), frequencies of total transitions ($F_{(9, 87)}$= 25.64, P=0.0024) and total running distance ($F_{(9, 87)}$= 37.61, P=0.0036) in the open field. In the BALB/SAL group, these behaviors were all significantly lower than those in the C57/SAL group (P=0.00017; P=0.0028; P=0.0026, respectively), whereas, no significant differences were found in the BALB/AVP group, BALB/DNQX group and BALB/NMDA group compared with the BALB/SAL group (AVP: P=0.165, P=0.678, P= 1.082, respectively; DNQX: P=0.356; P=0.182, P=0.176, respectively; NMDA: P=0.741, P=0.382, P=0.933, respectively).

Increases in the percentage of time spent in the central area, frequency of total transitions and total running distances were found in the BALB/AMPA group, BALB/MK-801 group, BALB/SSRI49415 group, ALB/AVP+MK-801 group, and BALB/SSRI49415+DNQX group when compared with the BALB/SAL group (AMPA: P=0.0038, P=0.021, P=0.0686, respectively; MK-801: P=0.0031, P=0.0031, P=0.0024, respectively; SSRI49415: P=0.0022, P=0.0031, P=0.0045, respectively; AVP+MK801: P=0.0016, P=0.0055, P=0.0064; SSRI49415+DNQX (SSDN): P=0.0057, P=0.0042, P= 0.0038, respectively) (Figure 1).

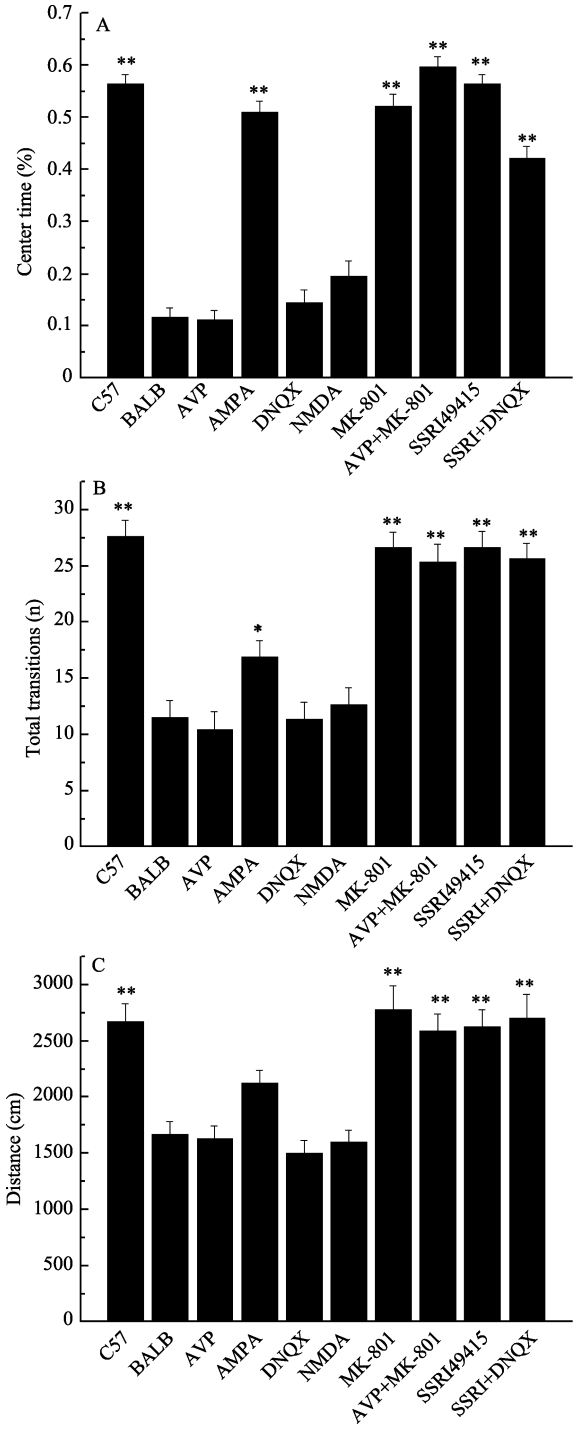

Figure 1 Behaviours of mice in the open field test

A: Percentage of time spent in central area; B: Total transitions; C: Total moving distance. * : $P<0.05$; ** $P<0.01$ (one-way ANOVA).

Elevated plus-maze

Results of the elevated plus-maze tests showed that drug treatments had significant effects on time spent in the open arms, frequency of transitions to each arm and total moving distance (time spent in the open arms: $F_{(9, 87)}$= 34.63, P=0.0016; transitions to each arm: $F_{(9, 87)}$=38.65, P=0.0041; total moving distance: $F_{(9, 87)}$=36.25, P= 0.0017). These behaviors in the BALB/SAL group were all significantly lower than those in the C57/SAL group (P=0.0034, P=0.0024, P=0.027, respectively); however, no differences among these behaviors were found when comparing the BALB/AVP group, BALB/ DNQX group and BALB/NMDA group with the BALB/SAL group (AVP: P=1.653, P=0.962, P=0.894, respectively; DNQX: P=0.866, P=1.033, P=0.886, respectively; NMDA: P=0.653, P=0.965, P=1.312, respectively).

However, increases in the percentage of time spent in open arms, frequency of total transitions and total running distances were found in the BALB/AMPA group, BALB/MK-801 group, BALB/SSRI49415 group, ALB/ AVP+MK-801 group, and BALB/SSRI49415+DNQX group compared with the BALB/SAL group (AMPA: P= 0.00085, P=0.0012, P=0.0837, respectively; MK-801: P=0.0018, P=0.0031, P=0.0034, respectively; SSRI4-9415: P=0.00046, P=0.0056, P=0.0038, respectively; AVP+MK801: P=0.00048, P=0.0057, P=0.028; SSRI49-415+DNQX (SSDN): P=0.0055, P=0.0076, P=0.0045, respectively) (Figure 2).

DICUSSION

Using the open field and elevated plus-maze tests, BALB mice were characterized with higher levels of anxiety-like behaviors, lower locomotor activities and lesser exploration compared with the C57 mice, which was consistent with previous findings (An & Tai, 2010). These results suggest that BALB mice could be applied in pharmacological and pathological studies as a high-anxiety animal model.

In this study, significant anxiolytic effects were found in mice treated with MK-801. When mice were under DNQX (blocker of AMPA receptors) treatment, their NMDA receptor expressions increased. Therefore, AMPA receptors and NMDA receptors were correlated and played an important role in the regulation of anxiety-like behavior. Although anxiolytic effects can be induced by AMPA receptor agonists and NMDA receptor antagonists, DNQX (AMPA receptors antagonist) and over-activation of NMDA receptors could result in increased anxiety and depression levels, as supported in our study.

Kole et al (2004) found that the expression of the NMDA receptor was markedly high in the brains of patients with anxiety. Matthews et al (1995) found that

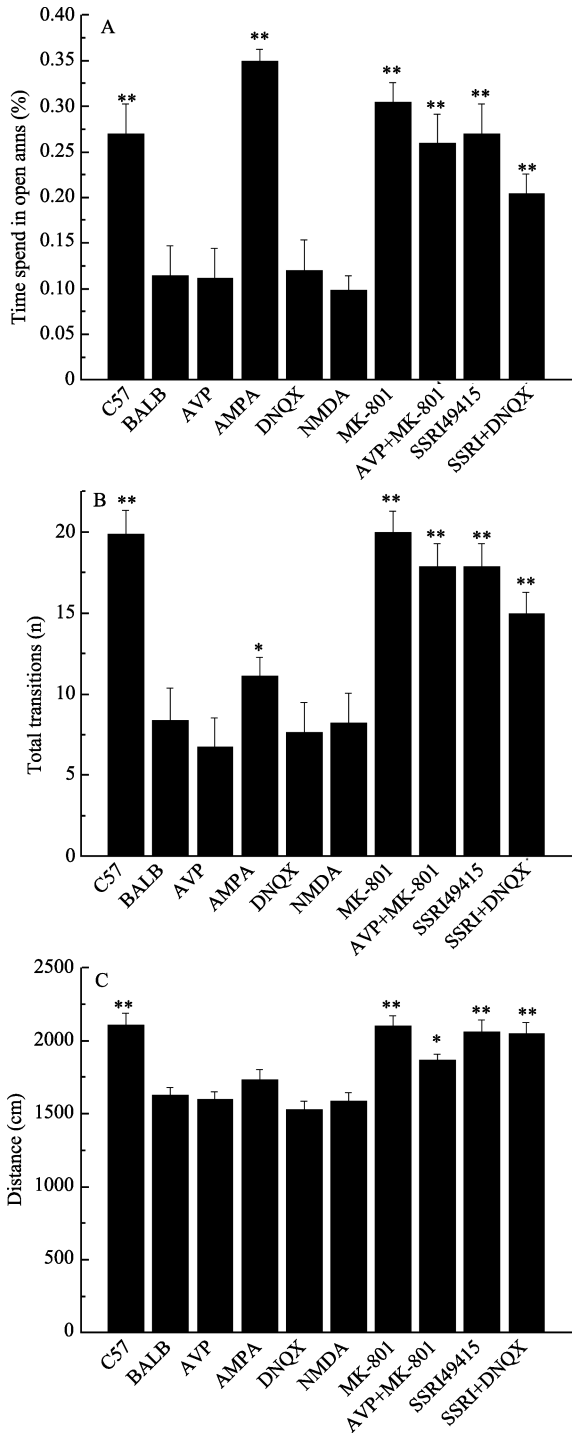

Figure 2 Behaviors of mice in the elevated plus-maze test

A: Percentage of time spent in open arms; B: Total transitions to each arm; C: Total moving distance. * : $P<0.05$; **: $P<0.01$ (one-way ANOVA).

although levels of the NMDA receptor and AMPA receptor in animals with anxiety or depression were altered obviously, their effects were antergic, i.e., BDNF expression increased with the activation of AMPA receptors to protect and nourish neurons, which is supported by our study. Other studies have claimed that the specific antagonist of the NMDA receptor, MK-801, can significantly increase the expressions of AMPA receptors, and thereafter, induce anxiolytic effects (Bergink et al 2004). Our results add further evidence that these two receptors interacted to regulate anxiety-like behaviors.

We found that BALB mice intracerebroventricularly microinjected with AVP displayed high levels of anxiety; however, when the V1aR antagonist, SSRI49415, was microinjected, anxiety levels were significantly reduced. These results are consistent with previous findings that AVP regulates emotions. Yayou et al (2008) reported that anxiety could be induced by activation of V1aR, whereas, anxiety levels could be reduced by the V1aR antagonist, SSRI49415. Appenrodt & Schwarzberg (2000) reported that anxiolytic effects could be found when V1aR was suppressed or knocked-out.

The Glu and AVP systems are both vital in the regulation of anxiety-like behaviors. However, previous research has mainly focused on the pharmacological mechanisms targeting stress-induced animals with high levels of anxiety. Thus, it is not yet clear whether the reported conclusions or hypotheses could be applied in BALB mice with innate high levels of anxiety-like behaviors. The neural mechanism underlying emotion adjustment is a complex synergic action executed by many neuronal chemical factors. In BALB mice, the interactions between Glu and AVP systems have not yet been reported.

Using adult male BALB mice as experimental subjects in this study, the vital roles of the NMDA receptor, AMPA receptor, and V1aR in the regulation of anxiety were confirmed through intracerebroventricular microinjection. Microinjection with AVP, NMDA and DNQX produced significant effects on anxiety in BALB mice, which may be based on the high innate anxiety level in BALB mice, and thus no dramatic changes were observed following treatment with anxiety-inducing drugs. However, significant anxiolytic effects were found when BALB mice were treated with AMPA, MK-801 and SSRI49415. Moreover, animals given drugs in combination, e.g. MK-801+AVP and DNQX+SSRI4-9415, also produced significant anxiolytic effects. These results are consistent with previous findings that stress-induced anxiety is due to the over-activation of the NMDA receptor or V1aR.

In our study, no antergic effects on anxiety-like

behaviors were found between V1aR and AMPA/NMDA receptors, suggesting that anxiety-like behaviors in BALB mice were associated with disorders of Glu and its AMPA/NMDA receptors, as well as AVP and V1aR. However, in BALB mice with high levels of anxiety-like behaviors, the effects induced by AMPA and NMDA receptors were different.

The anxiolytic effects induced by MK-801, AMPA and SSRI49415 treatments were more obvious compared with the anxiety-inducing effects found in AVP, NMDA and DNQX (AMPA receptors antagonist) treatment, suggesting that anxiety-like behaviors in BALB mice may be induced by changes in activity of the NMDA receptor and V1aR and deceasing activity in the AMPA receptor. We hypothesize that anxiety-like behaviors were induced by the NMDA receptor activating AVP release; therefore, the anxiolytic effects were induced by the NMDA receptor antagonist, MK801, which blocks the release of AVP (Hashimoto, 2011; Yamaguchi & Yamada, 2006). Whereas, the anxiolytic effects of the AMPA receptor were executed through suppressing AVP and V1aR. However, the possibility that anxiety could also be induced by AVP through V1aR via activating the NMDA receptor may not be excluded. The blocker of AVP receptor was possibly able to relieve anxiety by reducing the activities of Glu and NMDA in the brain (Joëls & Urban, 1984). However, the underlying mechanisms still need to be elucidated using other techniques.

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