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Research Article

Incoordination among subcellular compartments is associated to depression-like behavior induced by chronic mild stress

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Summary

Background: Major depressive disorder is characterized as persistent low mood. Chronic stressful life in genetic susceptible individuals is presumably major etiology that leads to dysfunctions of monoamine and hypothalamus-pituitary-adrenal axis. These pathogenic factors cause neuron atrophy in the limbic system for major depressive disorder. Cell-specific pathophysiology is unclear, to which we investigate prelimbic cortical GABAergic neurons and their interaction with glutamatergic neurons in depression-like mice.

Methods: The mice were treated by chronic unpredictable mild stress (CUMS) in three weeks until they expressed depression-like behaviors confirmed by sucrose preference, Y-maze and forced swimming tests. The structures and functions of GABAergic and glutamatergic units in prelimbic cortices were studied by cell imaging and electrophysiology in CUMS-induced depression mice versus controls.

Results: In depression-like mice, prelimbic cortical GABAergic neurons show incoordination among the subcellular compartments, such as the decreased excitability and synaptic outputs as well as the increased reception from excitatory inputs. GABAergic synapses on glutamatergic cells demonstrate the decreased presynaptic innervation and the increased postsynaptic responsiveness.

Conclusions: CUMS-induced incoordination in prelimbic cortical GABAergic and glutamatergic neurons dysregulates their target neurons, which may be pathological basis for depressive mood. The rebalance of compatibility among subcellular compartments would be ideal strategy to treat neural disorders.

Key words: depression, GABA, glutamate, neuron, prefrontal cortex and stress
Introduction

Major depressive disorder is characterized as anhedonia, low self-esteem and suicide. Its etiology is thought to be stressful environments plus genetic susceptibility (Camp and Cannon-Albright, 2005; Jabbi et al., 2008; Lohoff, 2010; Keers and Uher, 2012; Hamilton et al., 2013; Klengel and Binder, 2013; Moylan et al., 2013; Wilde et al., 2013). The sustained stress to the genetically vulnerable individuals leads to dysfunctions of monoamine, brain-derived neurotrophic factor and hypothalamus-pituitary-adrenal axis (Elhwuegi, 2004; Brunoni et al., 2008; Rohleder et al., 2010; Strekalova et al., 2011; Berton et al., 2012; Guo and Lu, 2014), which induce neuron atrophy in brain reward circuits, such as the prefrontal cortex, amygdala and hippocampus, in the depressive patients and stress animals (Bennett et al., 2008; Elizalde et al., 2008; Pittenger and Duman, 2008; C. H. Duman, 2010; Banasr et al., 2011; Lin and Sibille, 2013; Sandi and Haller, 2015). The brain includes the excitatory and inhibitory neurons. Their physiological coordination is critical for the neuron encoding to manage well-organized cognitions (Freund, 2003; Buzsaki et al., 2004; Ascoli et al., 2008). Cell-specific pathophysiology in major depressive disorder remains unclear (R. S. Duman and Aghajanian, 2012; Thompson et al., 2015).

In terms of the role of GABAergic neurons in major depressive disorders, immunocytochemistry in postmortem brain tissues from major depression subjects demonstrates the decrease of neuronal density in the prefrontal cortices (Sanacora et al., 2004; Rajkowska et al., 2007; Karolewicz et al., 2010; Maciag et al., 2010; Khundakar et al., 2011). Studies by imaging, biochemistry and gene analyses from the depression subjects indicate low GABAergic tone in the brain (Oruc et al., 1997; Torrey et al., 2005; Bajbouj et al., 2006; Hettema et al., 2006; Hasler et al., 2007; Price et al., 2009; Levinson et al., 2010; Croarkin et al., 2011; Plante et al., 2012; Veeraiah et al., 2014), despite argument (Godlewska et al., 2015). Therefore, the enhancers of GABA_A receptors are used as antidepressants, but there is controversy in therapeutic outcome (Petty et al., 1995; Smith et al., 2002; Kendell et al., 2005; Morishita, 2009; Luscher et al., 2011; Mohler, 2012). To the inconsistencies, we hypothesize that there are incompatible changes in the sub-compartments of GABAergic neurons and synapses, such as presynaptic GABA...
release versus postsynaptic GABA receptors and/or the outputs of GABAergic neurons versus their reception from excitatory inputs. We aim to examine depression-related pathology in the subcellular compartment of GABAergic neurons and their interaction with glutamatergic neurons in the medial prefrontal cortex. The elucidation of these issues provides new ideas for developing antidepressants in the manners of type-specific neurons and their subcellular compartments.

Pathophysiological changes in the prelimbic cortical GABAergic and glutamatergic neurons were examined in the mice of expressing depression-like behavior induced by chronic unpredictable mild stress (CUMS). Cortical GABAergic neurons and glutamatergic neurons in the mice were genetically labeled by green fluorescent protein and yellow fluorescent protein, respectively (G. Zhang et al., 2013). With their identifications, we were able to study mutual innervation between GABAergic and glutamatergic neurons by confocal cell imaging as well as their spike encoding and synapse dynamics by whole-cell recordings. With these analyses, we expect to reveal cell-specific pathology of major depressive disorders, especially subcellular incoordination.

Methods and Materials

All experiments were done in accordance with the guidelines and regulations by Administration Office of Laboratory Animals at Beijing China. All experimental protocols were approved by Institutional Animal Care Unit Committee in Administration Office of Laboratory Animals at Beijing China (B10831).

The mouse model of major depressive disorder induced by chronic unpredictable mild stress: In order to examine neuron-specific pathophysiology associated with major depressive disorders, we applied C57 Thy1-YFP/GAD-GFP mice whose GABAergic neurons and glutamatergic neurons were genetically labeled by green fluorescent protein (GFP) and yellow fluorescent protein (YFP), respectively (G. Zhang et al., 2013). The male mice were used starting at postnatal day 21. In week one for their adaptation to the experiments, their body weight, locomotion, sucrose preference and Y-maze test were measured to collect self-control data. The mice of showing the consistent values in these measurements were separated into...
two groups, chronic unpredictable mild stress (CUMS) and control, in order to reduce the variations among the mice. The control mice lived without the following stresses.

Based on depression risk factors, such as weaknesses in cognitive function, emotional regulation, social interaction skill, circadian and stress response (Southwick and Charney, 2012), we used chronic stress to produce depression-like mice in the following principle. The mice lived in stressful environment, made efforts to challenge these conditions and experienced defeat outcomes, which drove them to feel cognitive and emotional inabilities and in turn to be anhedonia and low self-esteem. The procedures for the CUMS mice include their adaptation, the CUMS and the behavioral tests (Figure 1A).

The stressful environments included social isolation, tilted cage, empty cage, damp sawdust cage, restraint space, white noise, strobe light and circadian disturbance (Willner, 2005; Schweizer et al., 2009; Strekalova et al., 2011; Berton et al., 2012; Hill et al., 2012). Except for the social isolation, these conditions were randomly selected to treat the mice in the manners of their separations or combinations every day. These treatments were applied about 1~14 hours in durations and 1~12 hours in intervals (Table 1). The durations and intervals were unpredictable to the mice. This CUMS was sustained for three weeks until some of the mice expressed anhedonia and low self-esteem. We did not use extreme stress in a single pattern, such as electrical shock, social defeat and tail clamp, since these protocols might induce the outcome similar to posttraumatic stressful disorder.

Whether the CUMS-treated mice in 3 weeks fell into anhedonia and low self-esteem was tested in day 29~31. The sucrose preference test (SPT) and Y-maze test (YMT) were used to assess the anhedonia, and the forced swimming test (FST) was used to estimate their self-esteem (Porsolt et al., 1978; Willner et al., 1987; Dellu et al., 1992; C. H. Duman, 2010; Overstreet, 2012). The SPT was done by 1% sucrose water versus water for four hours. The SPT value was presented as a ratio of the ingested sucrose water to the ingested sucrose water plus water. The YMT was performed by monitoring mouse staying in a special arm and other two arms for 2 minutes. The end of this special arm included a female mouse (named as M-arm). M-arm stay time was presented by a ratio of stay time in M-arm to that in three arms. The FST was
done by recording immobile time in a water cylinder (10 centimeters in diameters and 19 centimeters in water depth at 25±1°C). To quantify the FST, immobile time and latency (a period to mouse immobility in the first time) were presented. In these tests, the SPT was given once a week, the YMT was given before and after the CUMS and the FST was given one time after the CUMS. Before the SPT, the mice in the CUMS and control were deprived from food and water for 3 hours to drive their intension of drinking water. In the YMT, these arms were cleaned by 70% ethanol and then water after each test to reduce the effect of odor on the test. Carefulness in these tests was taken by performing them in a quiet room, no additional stresses, same circadian circle for all mice and their adaptation in the test environment.

An expression of depression-like behaviors was accepted if the mice in the CUMS group showed the decreases in sucrose preference (twice at ends of week two and three), M-maze stay time and latency, as well as the increase in immobile time, compared with these values during their self-control period (the first week) and in the control group of the mice. The mice with the significant changes in all of three tests were defined as CUMS-induced depression-like mice or depression-like mice. The CUMS-treated mice in 3 weeks met this criterion about 30%, implying their vulnerability to the stress. These mice were used as depression-like mice to study cell imaging and electrophysiology. It is noteworthy that some mice without any change in these three tests are considered as resilience, i.e., their invulnerability to stress situations, in our study. The mechanism underlying stress vulnerability and invulnerability is not our current topic that will be studied in the future. As 30% of CUMS-treated mice met the depression criteria and all CUMS-treated mice did not show a change of the SPT at the end of week one, the stressful situations in our study were thought to be mild stress.

**Brain slices and neurons.** To have more health brain cells for whole-cell recordings, we prepared cortical slices by the following procedures. The mice were anesthetized by isoflurane inhaling, and were infused by the artificial cerebrospinal fluid (ACSF) and oxygenated (95%O$_2$ and 5%CO$_2$) at 4°C into their left ventricles until the bodies became cold, in which the concentrations (mM) of the chemicals were 124 NaCl, 3 KCl, 1.2 NaH$_2$PO$_4$, 26 NaHCO$_3$, 0.5 CaCl$_2$, 4 MgSO$_4$, 10 dextrose and 220 sucrose at pH 7.35.
The mouse heads were immediately decapitated by guillotine and placed into this cold oxygenated ACSF with the brain isolation. The cortical slices (300 μm) in coronal direction were cut by Vibratome in this cold oxygenated ACSF. They were held in another oxygenated ACSF (124 NaCl, 3 KCl, 1.2 NaH₂PO₄, 26 NaHCO₃, 2 CaCl₂, 2 MgSO₄, 10 dextrose, and 5 HEPES, pH 7.35) at 25°C for 2 hours. Each slice was placed into a submersion chamber (Warner RC-26G) that was perfused by the oxygenated ACSF at 31°C for electrophysiological recordings (Jin-Hui. Wang and Kelly, 2001; Chen et al., 2008; Ge et al., 2014). The chemical reagents were from Sigma.

Whole-cell recording was done on GFP-labeled GABAergic and YFP-labeled glutamate neurons in layer III-IV of the prelimbic cortices under DIC-fluorescent microscope (Nikon FN-E600, Japan). The wavelength at 488 nm excited the fluorescence of GFP-labeled neurons, and that at 575 nm excited the fluorescence of YFP-labeled neurons. GABAergic neurons expressed fast spikes with less adaptation in their amplitude and frequency, the typical properties for the interneurons (Freund and Buzsaki, 1996; McKay and Turner, 2005; J. H. Wang et al., 2008; Lu et al., 2014). Glutamatergic neurons demonstrated the pyramidal somata and spike adaptation.

**Whole-cell recording and neuronal functions.** The neurons were recorded by MultiClamp-700B amplifier under voltage-clamp for their synaptic activity and the current-clamp for their intrinsic property. Electrical signals were inputted to pClamp-10 (Axon Instrument Inc.) for data acquisition and analysis. An output bandwidth of the amplifier was set at 3 kHz. The pipette solution of recording excitatory events included (mM) 150 K-gluconate, 5 NaCl, 5 HEPES, 0.4 EGTA, 4 Mg-ATP, 0.5 Tris-GTP, and 5 phosphocreatine (pH 7.35; (Ge et al., 2011; Yang et al., 2014). The solution for studying inhibitory synapses contained (mM) 130 K-gluconate, 20 KCl, 5 NaCl, 5 HEPES, 0.5 EGTA, 4 Mg-ATP, 0.5 Tris–GTP and 5 phosphocreatine (F. Zhang et al., 2012). These pipette solutions were freshly made and filtered (0.1 μm). The osmolarity was 295~305 mOsmol and pipette resistance was 5~6 MΩ.

The functions of GABAergic neurons were assessed including their active intrinsic properties and inhibitory outputs (J.-H. Wang, 2003). The inhibitory outputs were assessed by recording spontaneous
inhibitory postsynaptic currents (sIPSC) on glutamatergic neurons in the presence of 10 µM 6-Cyano-7-nitroquinoxaline-2,3-(1H,4H)-dione (CNQX) and 40 µM D-amino-5-phosphonovanolenic acid (D-AP5) in the ACSF to block ionotropic glutamatergic receptors. 10 µM bicuculline was washed onto the slices at the end of experiments for blocking sIPSCs to test that synaptic responses were mediated by GABA$_A$R. The pipette solution with a high concentration of chloride ions makes reversal potential to be -42 mV. sIPSCs are inward when membrane potential is held at -65 mV (Wei et al., 2004; F. Zhang et al., 2012).

The functions of excitatory neurons were evaluated based on their active intrinsic properties and excitatory output (J.-H. Wang, 2003). The excitatory outputs were assessed by recording spontaneous excitatory postsynaptic currents (sEPSC) on GABAergic neurons in the presence of 10 µM bicuculline in the ACSF to block GABA$_A$R (J.-H. Wang, 2003; Yu et al., 2012). 10 µM CNQX and 40 µM D-AP5 were added into the ACSF at the end of experiments to test whether synaptic responses were mediated by glutamate receptor, which blocked sEPSCs in our studies.

The recording of spontaneous synaptic currents, instead of evoked synaptic currents, is based on the following reasons. sEPSC and sIPSC amplitudes represent the responsiveness and the densities of postsynaptic receptors. The frequencies imply the probability of transmitter release from an axon terminal and the number of presynaptic axons innervated on the recorded neuron (Zucker and Regehr, 2002; Stevens, 2004). Such parameters can be used to analyze presynaptic and postsynaptic mechanisms as well as to compare them with morphological data about neuronal interaction. The evoked postsynaptic currents cannot separate these mechanisms. We did not use TTX into the ACSF to record miniature postsynaptic currents since we had to record neuronal excitability. As the frequency of synaptic activities was less than those of sequential spikes (Figures 2, 4~5) and spontaneous spikes were never recorded on the neurons in our cortical slices, sIPSCs and sEPSCs were not generated from spontaneous action potentials. Synaptic events in our recording are presumably miniature postsynaptic currents. This point is granted by a single peak of postsynaptic currents in our study.
Action potentials at the cortical neurons were induced by injecting the depolarization pulse. Their excitability was assessed by input-outputs (spikes versus normalized stimuli) when various stimuli were given (Chen et al., 2006). We did not measure rheobase to show cellular excitability, as this strength-duration relationship was used to assess the ability to fire single spike. We measured the ability of firing sequential spikes (J. H. Wang et al., 2008).

Data were analyzed if the recorded neurons had the resting membrane potentials negatively more than -60 mV, and action potential amplitudes more than 90 mV. The criteria for the acceptance of each experiment also included less than 5% changes in resting membrane potential, spike magnitude, and input resistance throughout each recording. The series and input resistances in all neurons were monitored by injecting hyperpolarization pulses (5 mV/50 ms), and calculated by voltage pulses versus instantaneous and steady-state currents.

*Cell imaging in the prelimbic cortex:* The mice were anesthetized by the intraperitoneal injection of urethane (1.5 g/kg), and perfused by 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS) into their left ventricle until their bodies were rigid. The brains were fixed in 4% paraformaldehyde for additional 24 hours. The cortical tissues were sliced in a series of coronal sections (100 μm). The images for glutamatergic neurons and GABAergic neurons in layers III~IV were photographed under confocal microscopy with oil lens (Plan Apo VC 60X, 1.4NA; Nikon A1R plus, Tokyo, Japan). Despite the peaks of GFP and YFP emission wavelength are 510 and 525 nm, respectively, we scanned GFP by setting the optical grate at 510 nm and YFP by the grate at 540 nm, respectively, to separate their images.

The processes of glutamatergic and GABAergic neurons were measured in each of the sections (Ni et al., 2010) by using ImageJ (version 1.47; National Institute of Health, USA). In terms of the structural interaction between excitatory and inhibitory neurons, we analyzed their mutual innervations by counting the contacts of presynaptic boutons on postsynaptic neurons. These contacts were counted from the layer-by-layer of confocal cell imaging, i.e., they were not the overlaps of three-dimension imaging. YFP-labeled glutamatergic axon boutons on GFP-labeled GABAergic neurons in contacts per neuron and
GFP-labeled GABAergic axon terminals on YFP-labeled glutamatergic dendrites in contacts per 100 μm length were counted (G. Zhang et al., 2013). It is noteworthy that fluorescent proteins are not labeled to all neurons due to low-efficient promoters. These low densities of neuronal contacts are parallel in control and depression-like mice.

Statistical analyses: The data of behavior tests, electrophysiology and morphology are presented as mean±SE. Paired t-test was used in the comparisons of experimental data before and after the CUMS in each of the mice. One-way ANOVA was used to make statistical comparisons in neuronal activity and morphology between control and depression-like groups.

Results

Chronic unpredictable mild stress induces the mice to express depression-like behaviors

The mice were treated by CUMS or control for three weeks. Their mood states were assessed by sucrose preference test (SPT), Y-maze test (YMT) and forced swimming test (FST). In the mice showing the significant changes in all of these tests, the SPT values are 48.58±5.1% in CUMS-treated mice (n=10), 80.1±2.2% before their CUMS treatment (self-control) and 86.62±2.8% in control mice (n=11, Figure 1B). The SPT values in CUMS-treated mice versus their self-control and control mice are statistical difference (p<0.001). The ratios of stay time in M-arm to stay time in total arms are 61.6±6.2% in CUMS-treated mice (n=10), 82.1±3.4% in their self-control and 68.2±3.7% in control mice (n=11; Figure 1C). These values are different for CUMS-treated mice vs. their self-control and control mice (p<0.01). In addition, the values of immobile time in the FST are 241.6±8 seconds in CUMS-treated mice (n=10) and 206.2±8.2 seconds in controls (left panel in Figure 1D; p<0.01, n=11). Latencies in the FST are 31.1±2.6 seconds in CUMS-treated mice (n=10) and 52.45±3.9 seconds in controls (right panel in Figure 1D; p<0.001, n=11). The mice that show significant changes in all of these parameters are thought to be depression-like mice.

The CUMS leads to the mice expressing depression-like behavior. Pathophysiological interaction between the excitatory and inhibitory neurons in the prelimbic cortex was investigated in depression-like
mice and controls. The outputs of GABAergic neurons were studied by analyzing spontaneous inhibitory postsynaptic currents (sIPSC) and axon innervations on their targeted glutamatergic neurons. The intrinsic property of GABAergic neurons was assessed by measuring their input-output curves. The receptions of GABAergic neurons were evaluated by analyzing their processes and glutamatergic terminals.

**The outputs decrease in the GABAergic neuron of the prelimbic cortex from depression-like mice**

The innervations from GABAergic axons to glutamatergic neurons were counted by GFP-labeled axonal terminals on YFP-labeled glutamatergic neuron in the prelimbic cortex (Figure 2A). Their contacts appear decreased in depression-like mice (Figure 2B). GFP-labeled axonal terminals on per 100 μm of YFP-labeled apical dendrites from glutamatergic neurons are 5.26±0.3 in depression-like mice (red bar in Figure 2C, n=64) and 7.02±0.34 in control mice (blue, p<0.001, n=60 apical dendrites). Depression-like behavior is associated with the decreased innervation from GABAergic axons onto glutamatergic neurons in the prelimbic cortex.

Lower sIPSC frequency and higher sIPSC amplitude appeared in depression-like mice (Figure 3). Figure 3B shows cumulative probability versus sIPSC amplitude from depression-like mice and control. Figure 3C shows cumulative probability versus inter-sIPSC interval from two groups of mice. The values of sIPSC amplitudes at 67% cumulative probability are 11.7±1.1 pA in depression-like mice (n=10 cells) and 7.28±0.6 pA in controls (n=10 cells; p=0.004). Inter-sIPSC-intervals at 67% cumulative probability are 1115±111 ms in depression-like mice (n=10) and 737±128 ms in control (n=10; p=0.03). Depression-like behavior is associated with an incompatible change at inhibitory synaptic efficacy, i.e., the decreased GABA release and the increased receptor responsiveness, in the prelimbic cortex. The decreases in both GABA release and presynaptic GABAergic innervations strengthen the reliability of our data.

**The excitability decreases in the GABAergic neurons of the prelimbic cortex from depression-like mice**

Figure 4 shows neuronal abilities to convert excitatory inputs into spikes. GABAergic neurons in depression-like mice appear lower capability to encode spikes (red trace in Figure 4A), compared with the control (blue). Figure 4B illustrates spikes versus normalized stimuli in these GABAergic neurons from
control mice (blue symbols) and depression-like mice (red). Input-output curve in GABAergic neurons (n=16) of depression-like mice shifts right-low, compared to that in controls (n=15; p<0.05). Depression-like behavior is associated with the decreased capability to convert excitatory inputs into digital spikes in the GABAergic neurons of the prelimbic cortex.

*Glutamatergic innervations increase on prelimbic cortical GABAergic neurons from depression-like mice*

Excitatory synaptic activity was recorded on GABAergic neurons (Figure 5). sEPSC frequencies appear higher in depression-like mice than controls (Figure 5A). Figure 5B shows cumulative probability versus sEPSC amplitudes in depression-like mice and controls. Figure 5C shows cumulative probability versus inter-sEPSC intervals in two groups of mice. sEPSC amplitudes at 67% cumulative probability are 9.81±1.3 pA from depression-like mice (n=11 neurons) and 10.58±1.4 pA from controls (n=9 neurons; p=0.69). Inter-sEPSC-intervals at 67% cumulative probability are 280±31 ms from depression-like mice (n=11) and 563±55 ms in control (n=9; p<0.001). Depression-like behavior is associated with the raised release of glutamates onto GABAergic neurons.

The innervation from glutamatergic axons onto GABAergic neurons was counted by YFP-labeled axonal terminals on GFP-labeled GABAergic neurons. As showed in Figure 6A~B, their contacts appear raised in depression-like mice. YFP-labeled axonal terminals on each GABAergic neuron are 7.07±0.4 from depression-like mice (red bar in Figure 6C, n=43 neurons) and 5.48±0.4 from controls (blue, p<0.01, n=43 neurons). Depression-like behavior is associated with the increased terminations from glutamatergic axons onto GABAergic neurons.

The reception of GABAergic neurons from synaptic inputs was also examined by measuring their processes. The number of their dendritic processes appears raised in depression-like mice (Figure 7A~B). Processes per GABAergic neuron are 6.1±0.15 in the depression-like mice (red bar in Figure 7C, n=70 cells) and 5.27±0.18 in controls (blue, p<0.001, n=76 cells). Depression-like behavior is associated with an increased receptive field in prelimbic cortical GABAergic neurons. The consistent changes in sEPSC
frequency, presynaptic glutamatergic innervation and receptive filed at GABAergic cells from depression-like mice strengthen our conclusion.

No change in excitability in the prelimbic cortical glutamatergic neurons from depression-like mice

Figure 8 illustrates the ability of glutamatergic neurons to convert excitatory inputs into spikes in the prelimbic cortex. Glutamatergic neurons in depression-like mice appear no change in excitability (red trace in Figure 8A), compared with controls (blue). Figure 8B shows spikes versus normalized stimuli in glutamatergic neurons from depression-like mice (red circles, n=28 neurons) and controls (blue squares, n=37). The CUMS does not influence the capability of glutamatergic neurons to convert excitatory inputs into spikes in the prelimbic cortex.

Discussion

The GABAergic neurons in the prelimbic cortex from depression-like mice possess the decreased inhibitory synapse output and excitability (Figure 2~4), as well as the increased reception from excitatory synapses (Figures 5~7). Although the bidirectional change in their outputs versus receptions appears to be compensatory homeostasis, this incoordination among subcellular compartments of GABAergic neurons may reduce their ability to regulate the downstream neurons. On the other hand, the increased sensitivity to inhibitory inputs (Figure 3) and the unchanged excitability in the glutamatergic neurons (Figure 8) may fade their excitatory output to drive the target cells. The incompatibility among subcellular compartments and incoordination between inhibitory and excitatory neurons may cause the dysfunction of the prelimbic cortex for major depressive disorder (Figure 9).

The dysfunction of GABAergic neurons is hypothetically a primary change to induce subcellular compartment incompatibility as well as inhibitory versus excitatory neuron incoordination, since they are vulnerable to the pathological factors (Akaike, 1995; J.-H. Wang, 2003; Luscher et al., 2011; J. H. Wang et al., 2015). For instance, the stress hormones affect the function of GABA_A receptors (Hu et al., 2010; Skilbeck et al., 2010; Gunn et al., 2011; Mody and Maguire, 2011) and reduce the density of GABAergic
neurons in prenatal period (Uchida et al., 2014). The chronic stress impairs the reversal potential and the
density of GABA receptor-channels (Quintero et al., 2011; Wislowska-Stanek et al., 2013; MacKenzie
and Maguire, 2015), and lowers GABAergic tones (Torrey et al., 2005; Hasler et al., 2007; Plante et al.,
2012; Seney et al., 2014). Together these with our data in the prelimbic cortex, a testable hypothesis is
that stress-impaired GABAergic neurons leads to subcellular unit incompatibility and neuronal
incoordination in major depressive disorder. Securing the GABAergic neurons in the limbic system to
reverse the subcellular changes remains to be examined.

The studies from depression patients indicate low GABAergic tone in the central nervous system
(Oruc et al., 1997; Torrey et al., 2005; Bajbouj et al., 2006; Hettema et al., 2006; Hasler et al., 2007; Price
et al., 2009; Levinson et al., 2010; Croarkin et al., 2011; Plante et al., 2012). By analyzing GABAergic
cells and synapses in the prelimbic cortex from depression-like mice, we observed the decreases in
GABAergic axon output and soma excitability (Figures 2~4). Interestingly, the reception from
glutamatergic axonal inputs increases in GABAergic neurons (Figures 5~7). These changes may be due to
that stress-induced primary dysfunction in GABAergic neurons initiates unknown mechanism to enhance
their sensitivity and reception from excitatory synapses, i.e., a compensatory homeostasis among
subcellular compartments for neuron survival (Chen et al., 2008). Moreover, a decrease of presynaptic
GABA release and an increase of postsynaptic GABA$_A$-receptor responses (Figure 3) imply homeostasis
within GABAergic synapses, which may explain a controversy in the use of GABA-receptor enhancers as
antidepressant. The compensatory changes among subcellular compartments tend to maintain functional
homeostasis in the GABAergic neurons and synapses. However, the incompatibility among subcellular
compartments and the incoordination between presynaptic and postsynaptic compartments make neuronal
interaction and synaptic transmission to be inefficient.

The increases in glutamatergic axonal terminations and actions in prelimbic cortical GABAergic
neurons are seen in depression-like mice treated by the CUMS for three weeks (Figures 5~6). This result
is consistent with recent reports that the potentiation of excitatory synapses on the activated neurons in the
prelimbic cortex is associated with learned helplessness (M. Wang et al., 2014) as well as that the stress induces the increases in the number of hippocampal pyramidal neurons (Stockmeier et al., 2004) and in the densities of postsynaptic glutamate NR2A-receptors and PSD-95 in lateral amygdala (Karolewicz et al., 2009). This excitatory effect may act onto prelimbic cortical GABAergic neurons to induce their excitotoxicity for cell pathophysiology associated with major depressive disorder. This point is supportive for recent findings that a low dose of NMDA-receptor antagonist ketamine improves depression patients resistant to typical antidepressants and reverses synaptic deficits in depression-like mice (R. S. Duman and Aghajanian, 2012; Zarate et al., 2013).

On the other hand, a few of studies present the different conclusions. The depression of excitatory synapses is seen on the parvalbumin neurons of the prefrontal cortex in learned helplessness mice evoked by extreme stress (Perova et al., 2015). The weakness of excitatory synapses mediated by AMPAR in the nucleus accumbens is associated with the depression mice induced by stress in 5 days (Lim et al., 2012). Restraint stress in seven days induces a decreased excitatory synaptic activity in the prefrontal cortical pyramidal neurons including both NMDAR and AMPAR components (Yuen et al., 2012). To these differences in the studies from animal models, we consider the following reasons. The different types of neurons, neuronal circuits and brain areas (such as positive versus negative circuits) may express different cellular changes to make the complicated signs in major depressive disorders. The procedures in the different stress patterns and periods may lead to the inconsistent pathological changes. Different from the studies by applying learned helplessness or restraint in 5~7 days, our CUMS procedure includes mild stresses for three weeks, in which the mice in the first week did not show depression-like behaviors. Their mood and cognition are likely based on neuronal plasticity mixed from learning, working memory and emotion deficiency, similar to stressful social life in depression patients.

Our studies demonstrate the incompatibility among subcellular compartments and incoordination between GABAergic and glutamatergic neurons in the prefrontal cortex from depression-like mice. These data imply that the incoordination among subcellular compartments constitute neural substrates for major
depressive disorder and the rebalance of their coordination should be considered as a therapeutic strategy, since the coordination and compatibility among subcellular compartments are present under physiological conditions (Chen et al., 2008; J. H. Wang et al., 2013). It is noteworthy that other diseases in the central nervous system, such as anxiety and epilepsy, are associated with subcellular incoordination (Liu et al., 2014; Lei et al., 2015; J. H. Wang et al., 2015; Wen et al., 2015). The reset of neuronal homeostasis by rebalancing subcellular compatibility and coordination should be ideal strategies for the treatment of neural disorders.

By using the mice with YFP-labeled glutamatergic and GFP-labeled GABAergic neurons, we are able to analyze type-specific cell pathology in their subcellular compartments and mutual interaction. The analyses by morphology and electrophysiology indicate that the changes in cell structures and functions are consistent. The studies with neuronal identification and mutual supportive data make our conclusions confident. In the prelimbic cortices from depression-like mice induced by chronic mild stress, the synaptic outputs and excitability of GABAergic neurons decrease, and the reception of GABAergic neurons from excitatory synapses increases. Stress-induced incompatibility among the subcellular compartments of the GABAergic neurons as well as the incoordination between GABAergic and glutamatergic neurons lead to imbalanced neural networks in the prelimbic cortex, which are the bases of depressive mood.

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References


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Figure Legends

Figure 1 Chronic unpredictable mild stress (CUMS) leads the mice to express depression-like behaviors. A) The procedures produce depression-like mice including the adaptation for a week, the CUMS for three weeks and the behavioral tests in three days. B) shows the SPT values (%) in the mice from the CUMS (red bar) and control group (blue). C) shows the ratios of stay time in M-arm to stay time in three arms by the YMT in the mice from the CUMS (red bar) and control (blue). D) shows immobile time (left panel) and latency (right) of staying in water cylinder by the FST in the mice from the CUMS (red bar) and control (blue). Two asterisks show p<0.01 and three show p<0.001, in which one-way ANOVA was used for the comparisons between the CUMS and control while paired-t test was for the comparisons before and after the CUMS.

Figure 2 Inhibitory axon innervations onto the glutamatergic neurons are downregulated in the prelicmic cortex from the depression-like mice. A) shows the area studied by morphology of neurons and synapses in the prelimic cortex (dash-line). B) illustrates the innervations of GABAergic axons (green) onto the apical dendrites of glutamatergic neurons (yellow) in a control mouse (left panel) and in a depression-like mouse (right panel). C) illustrates the comparisons of innervations per 100 µm dendrite from depression-like mice (red bar, n=64 apical dendrites) and controls (blue, n=60 apical dendrites; asterisks, p<0.001).

Figure 3 Inhibitory synaptic transmission is downregulated in the glutamatergic neurons of the prelimbic cortex from depression-like mice. sIPSCs were recorded under voltage-clamp in the brain slices from the control and depression-like mice in presence of 10 µM CNQX and 40 µM D-AP5. A) Left panels show sIPSCs from a control mouse (blue traces) and right panels illustrate sIPSCs from depression-like mouse (reds). Calibration bars are 20 pA in vertical bar as well as 2 seconds (top traces) and 90 milliseconds (bottoms) in horizontal. B) shows cumulative probability versus sIPSC amplitudes from the depression-like mice (red circle symbols) and control (blue square symbols). Dash-lines indicate sIPSC amplitudes at
cumulative probability to 67% (CP_{67}) in the control (blue line; n=10) and in depression-like mice (red; n=10, p<0.01). C) illustrates cumulative probability versus inter-sIPSC intervals from the depression-like mice (red circle symbols) and control (blue square symbols). Dash-lines indicate inter-sIPSC intervals at cumulative probability to 67% (CP_{67}) in control (blue line; n=10) and in depression-like mice (red; n=10, p<0.05).

**Figure 4** The ability to produce the sequential spikes on the GABAergic neurons of the prelimbic cortices decreases in the depression-like mice. The sequential spikes induced by various stimulus intensities were recorded on the GABAergic neurons in cortical slices under current-clamp. A) illustrates depolarization-induced the sequential spikes on the GABAergic neurons from a depression-like mouse (red trace) and a control mouse (blue trace). B) shows spikes per second versus normalized stimuli in GABAergic neurons from the depression-like mice (red circle symbols, n=16 neurons) and the controls (blue square symbols, n=15 neurons). An asterisk indicates p<0.05. Arrow indicates spikes versus stimulus intensity taken for panel A.

**Figure 5** The frequency of excitatory synaptic transmission is upregulated in the GABAergic neurons of the prelimbic cortex from depression-like mice. sEPSCs were recorded under voltage-clamp from control and depression-like mice in the presence of 10 μM bicuculline. A) Left panels illustrate sEPSCs from a control mouse (blue traces) and right panels show sEPSCs from depression-like mouse (reds). Calibration bars are 20 pA as well as 2 seconds (top traces) and 90 ms (bottoms). B) illustrates cumulative probability versus sEPSC amplitudes from the depression-like mice (red circle symbols) and control (blue squares). Dash-lines indicate sEPSC amplitudes at cumulative probability to 67% (CP_{67}) in the control (blue line; n=9) and in depression-like mice (red; n=11, p=0.69). C) illustrates cumulative probability versus inter-sEPSC intervals from depression-like mice (red circle symbols) and control (blue squares). Dash-lines are
inter-sEPSC intervals at the cumulative probability to 67% (CP_{67}) in the control (blue line; n=9) and in the depression-like mice (red; n=11, p<0.001).

**Figure 6** Excitatory axon innervation onto the GABAergic neurons is upregulated in the prelimbic cortex from the depression-like mice. **A**) shows the innervations of glutamatergic axons (yellow) onto the soma of GABAergic neuron (green) in a control mouse. **B**) shows the innervations of the glutamatergic axons (yellow) onto the soma of the GABAergic neuron (green) in a depression-like mouse. **C**) illustrates the comparisons of innervations per neuron from depression-like mice (red bar, n=43 neurons) and controls (blue, n=43 neurons; asterisks, p<0.01).

**Figure 7** The dendritic processes on the GABAergic neurons are upregulated in the prelimbic cortex from the depression-like mice. **A**) illustrates a GABAergic neuron and its processes from a control mouse. **B**) shows a GABAergic neuron and its processes from a depression-like mouse. **C**) shows the comparison of processes per GABAergic neuron from depression-like mice (red bar, n=70 neurons) and controls (blue, n=76 neurons; asterisks, p<0.001).

**Figure 8** The ability to fire the sequential spikes on the glutamatergic neurons of the prelimbic cortices does not change in the depression-like mice. The sequential spikes induced by various stimulus intensities were recorded on GABAergic neurons in cortical slices under current-clamp. **A**) shows depolarization-induced sequential spikes on glutamatergic neurons from a control mouse (blue trace) and a depression-like mouse (red trace). **B**) shows spikes per second versus normalized stimuli in glutamatergic neurons from the depression-like mice (red circle symbols, n=28 neurons) and the controls (blue squares, n=37 neurons). Arrow indicates spikes versus stimulus intensity taken for panel A.
**Figure 9** Incompatible alternations occur in the GABAergic neurons and glutamatergic neurons of the prelimbic cortices from depression-like mice. In GABAergic neurons (round), their intrinsic property and synaptic outputs decrease (blue). Their receptions from excitatory synaptic transmission and innervations as well as their receptive fields increase (red). The incompatibility among the subcellular compartments of GABAergic neurons reduces their efficiency to coordinate their downstream neurons. In glutamatergic neurons (pyramidal), their responses to GABAergic inputs increase, their spiking capability does not alter and their excitatory outputs increase. These incompatible changes among the subcellular compartments of glutamatergic neurons attenuate their efficiency to program the neural codes. Together these changes, the interactions between GABAergic and glutamatergic neurons are deteriorated.
Table 1 Mild stresses and their applications to induce major depressive disorder in mice

<table>
<thead>
<tr>
<th>Mild stimulations</th>
<th>Definition</th>
<th>Durations</th>
<th>Intervals</th>
<th>Intensity</th>
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<td>Social isolation</td>
<td>Living in a cage alone</td>
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<td>1–2 days</td>
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<td>Tilted cage</td>
<td>Staying in 45°-slanted cage with 50 ml water in its low end</td>
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<td>Damp sawdust cage</td>
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<td>White noise</td>
<td>Audible hissing sound</td>
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<td>1–3 days</td>
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<td>Strobe light</td>
<td>Flashes at 2.5 Hz</td>
<td>6–14 hours per time</td>
<td>2–3 days</td>
<td>500 lux</td>
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<tr>
<td>Circadian disturbance</td>
<td>Living with irregular illumination</td>
<td>3 weeks</td>
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Abbreviations: dB, decibel; SI, social isolation; EC, empty cage; TC, tilted cage; DSC, damp sawdust cage; RS, restraint space; WN, white noise; SL, strobe light; CD, circadian disturbance; SPT, sucrose preference test; YMT, Y-maze test; FST, forced swimming test.
Figure 1

A) Adaptation
- Empty cage
- Tilted cage
- White noise
- Restraint space

B) Chronic unpredictable mild stress (CUMS)
- Damp sawdust cage
- Strobe light
- Social isolation
- Circadian disturbance

Behavioral Tests
- d28, d29: Sucrose preference test (SPT)
- d30: Y-maze test (YMT)
- d31: Forced swimming test (FST)

B) SPT
- Week 0, Week 1: Before CUMS
- After CUMS

C) YMT
- Time ratio of M-arm vs. three arms (%)
- Week 0, Week 3: Before CUMS
- After CUMS

D) FST
- Immobility time (seconds)
- Latency (seconds)
Figure 3

A) sIPSC from a control mouse and a depression-like mouse. The traces show differences in sIPSC amplitudes and frequencies.

B) Cumulative probability of sIPSC amplitudes for control and depression-like mice. The graph indicates a shift in distribution with depression-like mice having lower amplitudes.

C) Cumulative probability of inter-sIPSC intervals for control and depression-like mice. The graph shows a change in the frequency distribution with depression-like mice having longer intervals.
Figure 4

A

Membrane Potentials (mV)

Sequential spikes from a control mouse
Sequential spikes from a depression-like mouse

B

Spikes per Second vs. Normalized Stimuli

Control mice
Depression-like mice
Figure 6

A  B  C

Control mouse  Depression-like mouse

Number of innervations per interneuron

Control mice  Depression-like mice

**
Figure 7

A  B
Control mouse  Depression-like mouse

C

Processes per Cell

Control mice  Depression-like mice

**
Figure 8

A

Membrane Potentials (mV)

Sequential spikes from a control mouse
Sequential spikes from a depression-like mouse

B

Spikes per Second

Control mice
Depression-like mice

Normalized Stimuli