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Title: Neurovascular coupling under chronic stress is modified by altered GABAergic interneuron activity

Abbreviated title: Neurovascular coupling under chronic stress

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Abstract

Neurovascular coupling (NVC), the interaction between neural activity and vascular response, ensures normal brain function by maintaining brain homeostasis. We previously reported altered cerebrovascular responses during functional hyperemia in chronically-stressed animals. However, the underlying neuronal-level changes associated with those hemodynamic changes remained unclear. Here, using *in vivo* and *ex vivo* experiments, we investigate the neuronal origins of altered NVC dynamics under chronic stress conditions in adult male mice. Stimulus-evoked hemodynamic and neural responses, especially beta and gamma-band local field potential activity, were significantly lower in chronically-stressed animals, and the NVC relationship, itself, had changed. Further, utilizing acute brain slices, we discovered that the underlying cause of this change was dysfunction of neuronal nitric oxide synthase (nNOS)-mediated vascular responses. Using *in situ* hybridization (FISH) to check the mRNA expression of several GABAergic subtypes, we confirmed that only nNOS mRNA was significantly decreased in chronically-stressed mice. Ultimately, chronic stress impairs NVC by diminishing nNOS-mediated vasodilation responses to local neural activity. Overall, these findings provide useful information in understanding NVC dynamics in the healthy brain. More importantly, this study reveals that impaired nNOS-mediated NVC function may be a contributory factor in the progression of stress-related diseases.

Significance Statement

The correlation between neuronal activity and cerebral vascular dynamics is defined as neurovascular coupling, which plays an important role for meeting the metabolic demands...
of the brain. However, the impact of chronic stress, which is a contributory factor of many
cerebrovascular diseases, on NVC is poorly understood. We, therefore, investigated the
effects of chronic stress on impaired neurovascular response to sensory stimulation and their
underlying mechanisms. Multimodal approaches, from in vivo hemodynamic imaging and
electrophysiology to ex vivo vascular imaging with pharmacological treatment, patch-clamp
recording, FISH and immunohistochemistry (IHC) revealed that chronic stress-induced
dysfunction of nNOS-expressing interneurons contributes to NVC impairment. These
findings will provide useful information to understand the role of nNOS-interneurons in NVC
in normal and pathological conditions.

Introduction

Chronic stress is a known causal factor in the progression of major depressive
disorder (Dolan et al., 1994; Ota et al., 2014). Physical and psychological stressors affect the
brain system, particularly through activation of the hypothalamus-pituitary-adrenal (HPA)
axis, which results in glucocorticoid production (McEwen et al., 2015). Repeated exposure to
glucocorticoids affects dendritic remodeling and synaptic changes (Seib and Wellman, 2003;
Liston et al., 2006; Shansky et al., 2009). These neural alteration affects neuronal networks
and causes functional alterations such as sensory perception (Khasar et al., 2008; Zheng et al.,
2015) and sensory gating circuit in chronically-stressed animals (Stevens et al., 2001;
Maxwell et al., 2005). One of the chronic stress-associated neuronal alterations that is widely
studied is GABAergic interneuron dysfunction. In the medial prefrontal cortex (mPFC) and
hippocampus, both the number of GABAergic interneurons and their spontaneous inhibitory
postsynaptic currents (sIPSCs) were reduced after exposure to chronic stress (Hu et al., 2010; Holm et al., 2011; Gilabert-Juan et al., 2013). Since repeated stress exposure also influences the cardiovascular system (Low et al., 2009), recent studies have focused not only on changes in the neural network system but also on alterations in neurovascular coupling (NVC) under chronically-stressed conditions. In one instance, the malfunction of inwardly-rectifying potassium (K\text{ir}) channels in parenchymal arteriolar myocytes was observed in chronically-stressed mice (Longden et al., 2014). Moreover, our previous work with chronically-stressed rats revealed reduced cerebrovascular response following sensory stimulation (Lee et al., 2015).

Neurovascular coupling (NVC) — the tightly linked relationship between local neural activity and subsequent changes in cerebral blood flow (CBF) — is an important function for maintenance of brain homeostasis. NVC enables the microvascular system to meet the metabolic demands created by neural activity (Hillman, 2014). Excitatory neurons, inhibitory neurons, glia and cerebral vasculature are all components of the neurovascular coupling unit. Elaborate interplay within these units regulate adequate blood and nutrient supply to localized brain regions (Iadecola, 2017). For example, following the activation of NMDA receptors, pyramidal cells cause an increase in CBF via the COX-2-mediated prostaglandin E2 (PgE2) pathway (Lacroix et al., 2015). In addition to pyramidal cells, GABAergic interneurons have direct contact with nearby parenchymal arterioles. GABAergic interneurons regulate nearby arteriolar diameter through their expression of vasoactive mediators, such as neuropeptide Y (NPY), somatostatin (SOM), and/or neuronal nitric oxide synthase (nNOS) (Cauli et al., 2004; Kocharyan et al., 2008). Optogenetic activation of GABAergic interneurons can provoke an increase in CBF (Uhlirova et al., 2016; Vazquez et
al., 2018). These studies imply that any abnormal interactions between GABAergic interneuron activity and vascular functions under pathological conditions may alter hemodynamic signals. Thus, we can speculate that altered GABAergic neuronal function in a chronically-stressed brain will affect both overall hemodynamics and NVC.

Previously, we reported that chronically-stressed rats exhibit a decrease in cerebral blood volume (CBV) response to a sensory stimulus (Lee et al., 2015). From these observations, we hypothesized that there would be a neuronal activity-originated source that would induce such changes on neurovascular coupling and modulate vascular tone and function. To explore this hypothesis, we chose primary somatosensory cortex to adopt a validated experimental design from a wide range of neurovascular coupling imaging research (Berwick et al., 2008; Jordanova et al., 2015). In the present study, we investigate the underlying mechanisms of chronic stress-induced alterations in the neurovascular coupling relationship. Here, we propose that altered GABAergic interneuron activity, specifically in nNOS-positive interneurons, is the key component in altered neurovascular function observed after chronic stress exposure.

Materials and Methods

Experimental design. All experiments were done with C57BL/6N male mice. A total of 240 mice were included in the study: 20 mice (10 control and 10 stressed mice) for in vivo OIS imaging and LFP recording experiments, 6 mice (3 control and 3 stressed mice) for blood pressure measurements, 22 mice (12 control and 10 stressed mice) for FISH to measure mRNA expression of each subtype of GABAergic interneuron, 23 mice (12 control and 11
Animals. 8-week old healthy male C57BL/6N mice (OrientBio Inc., South Korea) were used. Mice were raised in a cage with *ad libitum* access to food and water. The environment was maintained with a 12-hour light/dark cycle (light on 9:00 A.M), 24-25 °C temperature, and 50-60% humidity. All experimental procedures were approved by the Institutional Animal Care and the Use Committee (IACUC) of Sungkyunkwan University.

Mouse model of chronic restraint stress. Chronically-stressed mouse models were established via the application of chronic restraint stress. This well-established protocol is known to induce depressive-like behaviors (Buynitsky and Mostofsky, 2009). Three weeks of restraint stress was applied to 8-week-old C57BL/6 mice. The mice were immobilized with plastic bags (Decapicones, Braintree Scientific Inc., MA, USA) in their home cages for 6 hours per day, starting at 10:00 A.M. During the 6-hour restraint period, animals were restricted from food and water intake. The control group mice were allowed to move freely in their individual cages. Each animal’s weight and food intake were checked every week.

Elevated plus maze (EPM) test. For behavioral phenotyping, we performed an elevated plus
maze (EPM) test at a day after the 3-week restraint stress protocol. EPM test is often used to measure anxiety-like behavior in stress models (Carobrez and Bertoglio, 2005). The plus maze consisted of four arms (30 cm x 5 cm), two opposite arms were enclosed by 20-cm walls (closed platform) and the other two arms were not enclosed by walls (open platform). Animal movement on the platforms was recorded for 5 min with a video recording and analyzing system (Ethovision XT, Noldus, Wageningen, Netherlands). The time each mouse spent in the open and closed arms was calculated automatically with behavior analysis software. For this experimental study, stress-resilient mice were excluded.

Blood sampling and enzyme-linked immunosorbent assay (ELISA). After the end of 3-weeks of stress induction, plasma was collected without stress exposure on the day of collection. Mice (control, n = 25; stress, n = 25) were briefly anesthetized with 3 % isoflurane (Hana Pharm Co., South Korea) using an anesthesia machine (VetEquip Inc., Livermore, CA), and trunk blood was collected in heparin-coated tubes (BD Vacutainer, Becton Dickinson, NJ, USA). Blood samples were centrifuged at 5000 rpm for 10 min. The concentration of corticosterone in the plasma was analyzed using a corticosterone ELISA kit (Assaypro LLC, MO, USA). Assay procedures were followed according to the instructions provided in the kit. The absorbance at a wavelength of 450 nm was scanned by a microplate reader (Synergy HT, BioTek, VT, USA). A standard curve was generated using standard solutions, and the sample concentrations were determined from the standard curve.

Blood pressure measurement. Blood pressure was measured using a tail-cuff blood pressure
measurement system (CODA, Kent Scientific Corp, CT, USA). Anesthesia was induced with 3% isoflurane and maintained with 1.3 mg/kg urethane (U2500, Sigma-Aldrich, MO, USA) to achieve a state similar to OIS imaging and LFP recording experiments. Body temperature was maintained at 37 °C using a homeothermic heating pad system (FHC Inc., ME, USA). Systolic, diastolic, and mean blood pressure of individuals were calculated as an average of 30-cycle measurements.

Animal surgery for in vivo experiments. For simultaneous measurement of hemoglobin-based optical intrinsic signal (OIS) and neural activity, we performed a 3 mm diameter craniotomy on an area over a region corresponding to forelimb response in the right primary somatosensory cortex (right S1FL). Animals were anesthetized with isoflurane using an anesthesia machine (VetEquip Inc., CA, USA). 3% isoflurane was used for induction, and the isoflurane level was maintained at 1.5% during the surgery. For head fixation, a customized chamber frame was mounted on the skull. A dental resin wall was built around the site of craniotomy. Body temperature was maintained at 37-38 °C using a homeothermic heating pad system (FHC Inc., ME, USA). After the surgery, the mice were anesthetized with urethane (1.3-1.4 mg/kg, i.p.) during OIS imaging and electrophysiology experiments. The use of anesthesia was intended to avoid acute stress effects on neurovascular responses caused by head fixation during CBV-weighted optical imaging and electrophysiological recordings. Urethane is known to induce a highly stable state of anesthesia (Boorman et al., 2015). It also preserves both excitatory and inhibitory synaptic transmission (Sceniak and MacIver, 2006) and neurovascular coupling (Berwick et al., 2008) with relatively limited effect on the cardiovascular system (Janssen et al., 2004). Throughout experiments we monitored animal
anesthesia level with the toe pinching test and indicators of physiological status (PhysioSuite, Kent Scientific Corp, CT, USA).

Optical intrinsic signal (OIS) imaging. Hemodynamic response to electrical forelimb stimulation was recorded with an optical imaging system (Imager 3001-Celox, Optical Imaging Ltd., Rehovot, Israel). The exposed cortex was filled with silicone oil (Sigma-Aldrich, MO, USA) and illuminated with an LED lamp (CLS150, Leica Microsystems CMS GmbH, Mannheim, Germany). Images were collected with a 10 Hz frame rate using a fast acquisition camera (Photonfocus AG, Lachen, Switzerland) through 50 mm tandem lenses. Since 546 nm is the isosbestic wavelength in the absorption spectra of oxyhemoglobin and deoxyhemoglobin, reflected light was filtered with a 546 ± 30 nm bandpass filter to measure the total amount of hemoglobin (Hbt) (Horecker, 1943). The pixel resolution of the field of view was 372 × 372 pixels, and the pixel size was 8 μm.

For electrical forelimb stimulation, two custom needle electrodes were placed in the left forelimb between digits 2 and 4. The stimulus was applied for 20 s using a stimulation generator (Master-9, A.M.P.I., Jerusalem, Israel) and consisted of 500 μs electrical pulses at 4 Hz frequency with 0.5 mA amplitude. The activated area within the right S1FL region was identified; then, the insertion site of the electrode for electrophysiology was decided. The inter-trial interval was 100 s, and the trials were repeated for 18 times per animal (control, n = 10; 3-week stress model, n = 10).

Intensity changes were computed for each trial with the baseline defined as the 5-second period preceding the stimulus onset. The region of interest (ROI) was set as the 5 × 5
pixels nearest to the position of the electrode tip avoiding surface vessels. Time series data was temporally averaged to each time point. The peak amplitude was determined within the stimulation period. The onset time was determined to be the time point at which the intensity change exceeded the baseline average by two standard deviations (SD). The summated image of 18 trials, representing one animal, was binned by 4 × 4 pixels. Pixel counts within the top 50% of the value of intensity change were used to calculate the spatial extent of the active area.

**Local field potential (LFP) recording.** An electrode with ~ 0.5 MΩ impedance (FHC, Inc., ME, USA) was placed within the activation area of the right S1FL region at a depth of 300 μm, targeting layer II/III of the somatosensory cortex. Electrophysiological activity during forelimb stimulation was recorded at 40 kHz frequency (Plexon Inc., TX, USA). The raw electrophysiology data was filtered between 0.5 and 200 Hz for LFP. The amplitudes of the LFPs induced by each electrical pulse were summed over 80 electrical pulses during a 20-second period of stimulation, then averaged for repeated trials. LFP analyses were performed by using the open-source Chronux 2.12 toolbox. LFP spectra were calculated by using five tapers and 1-second time windows with a step size of 50 ms. The frequency ranging from 2 to 4 Hz was taken to be the delta band, 4 to 7 Hz to be the theta band, 7 to 13 Hz to be the alpha band, 13 to 30 Hz to be the beta band, and 30 to 100 Hz to be the gamma band. The frequency ranging from 55 to 65 Hz was excluded from all analyses due to a notch filter at 60 Hz.
Brain slice preparation. Mice were decapitated after isoflurane anesthesia. The brains were quickly removed and placed into ice-cold artificial cerebrospinal fluid (ACSF) containing 93mM NMDG, 2.5mM KCl, 1.2mM NaH2PO4, 30mM NaHCO3, 20mM HEPES, 25mM Glucose, 5mM sodium ascorbate, 2mM Thiourea, 3mM sodium pyruvate, 10mM MgSO4, and 0.5mM CaCl2, adjusted to 300–310 mOsm, 7.4 pH and continuously bubbled with 95% O2:5% CO2. Coronal slices (300 μm thick) containing the somatosensory cortex were prepared using a vibratome (VT 1200S, Leica Biosystems, Wetzlar, Germany). Slices were then incubated at room temperature for at least 1h in oxygenated ACSF containing 124mM NaCl, 2.5mM KCl, 1mM NaH2PO4, 24mM NaHCO3, 10mM Glucose, 1mM sodium ascorbate, 2mM sodium pyruvate, 2mM MgSO4, and 2mM CaCl2. Slices were then transferred to a submerged recording chamber circulated with oxygenated ACSF using a peristaltic pump (ISMATEC, Wertheim, Germany). All *ex vivo* experiments were conducted under the condition of 95% O2. It is noted that although the condition of 20-30% O2 is recommended for young mice (Gordon et al., 2008; Hall et al., 2014; Mehina et al., 2017), our preliminary studies showed that our adult brain slices under a condition of 20-30% O2 did not well exhibit pre-constriction of penetrating arterioles by U46619. Hence, the protocol utilizing 95% O2 was chosen for our adult brain slice studies.

Vessel imaging in brain slices. Penetrating arterioles (8-14 μm in luminal diameter) in layer II/III of the somatosensory cortex were selected under infrared (IR) illumination, using a 63 x 0.9 NA water objective lens (Leica, Wetzlar, Germany). Vascular dynamic images were acquired every second on a Leica microscope (DM6000 FS, Leica, Wetzlar, Germany) with a digital CMOS camera (ORCA-Flash4.0, Hamamatsu, Shizuoka, Japan) at room temperature.
The pixel resolution of the field of view was 2048×2048 pixels with pixel size measuring 0.08 μm. Since acute brain slices have no vascular tonic movement, blood vessels were pre-constricted by circulating a thromboxane A2 receptor agonist, 9,11-dideoxy-9α,11α-methanoepoxyprostaglandin F_{2α} (U46619; 65-100 nM, Cayman, MI, USA). As the degree of vasoconstriction has been reported to influence the amplitude and polarity of vascular responses, only arterioles developing a similar and stable vasoconstriction (25-30% constriction level) within the 20 min following U46619 application were included in the analysis. Electrical stimulation used a stimulation generator (Master-9, A.M.P.I., Jerusalem, Israel) and consisted of 300 μs electrical pulses at 20 Hz frequency with 2V amplitude.

**Vascular reactivity analysis in brain slices.** Before the quantification of vascular luminal diameter changes, we used ImageJ (RRID:SCR_002285) for image rotation, Gaussian filtering, registration and generating SD maps. Specifically, all stack images were rotated to align vascular orientation vertically. Then, to remove red blood cell (RBC) signal present in the vascular lumen, we applied a temporal Gaussian filter with a 9-slice FWHM kernel. After correcting for the RBC signal, all images were realigned using the “StackReg” plug-in in Fiji to compensate for potential in-plane movements. Finally, using the filtered and realigned image, we generated a temporal SD map to find a stably changing luminal area during stimulation.

Next, the changes of luminal diameter were quantified from a registered image using a custom analysis method developed in MATLAB (MathWorks, MA, USA, RRID:SCR_001622). First, we selected three lines perpendicular to the vascular lumen. The perpendicular line selection was determined under two conditions: (1) the line should be ...
located at the stably changing luminal regions as determined by the SD map and (2) the distance between each line should be at least 10 μm to avoid selecting vascular lumen controlled by identical smooth muscles. For each selected line, an average line was recalculated by averaging the values within the area 0.4 μm above and below each line. A 2D line stack image was created by evolving the averaged line across all frames. To sum up, the three lines perpendicular to the vascular luminal area are arranged in temporal order to create three line-stack images for every vascular image stack.

The generated 2D line stack image exhibits vascular luminal boundaries on both sides along the temporal axis. Therefore, calculating the distance between the two defined luminal boundaries allows for measurement of the diameter change of vascular lumen over time. In order to find the coordinates of the luminal boundaries, we performed an “edge contrast enhancement” for clarified boundary contrast. Then, we found two coordinates which have “local minima” near each luminal boundary. In this way, we defined the coordinates at all temporal axes and quantified the distance between the luminal boundaries over time. We then converted the quantified diameter change into percent change using the initial baseline of the first minute.

Whole-cell recording. Whole-cell recordings of pyramidal neurons in the somatosensory cortex were acquired in acute coronal brain slices. Borosilicate glass pipettes (BF100-58-10, Sutter Instrument, CA, USA) with resistances ranging from 5-8 MΩ were pulled using a laser micropipette puller (P-1000, Sutter Instrument, CA, USA). For spontaneous EPSCs, the pipette was filled with an internal solution containing 135mM K-gluconate, 4mM KCl, 2mM NaCl, 10mM HEPES, 4mM EGTA, 4mM Mg-ATP, 0.3mM Na-GTP, pH adjusted to 7.2
with KOH (278-285 mOsmol). For spontaneous IPSCs, the pipette was filled with an internal solution containing 135mM CsCl, 4mM NaCl, 0.5mM CaCl2, 10mM HEPES, 5mM EGTA, 2mM Mg-ATP, 0.5mM Na2-GTP, 10mM QX-314, of which the pH had been adjusted to 7.2 with CsOH (278-285 mOsmol). During voltage clamp experiments, neurons were clamped at either −70 mV or 0 mV to measure EPSCs or IPSCs, respectively. Whole-cell voltage-clamp recordings were performed using a MultiClamp 700B amplifier (Molecular Devices, CA, USA), filtered at 2 kHz and digitized at 20 kHz using a Digidata 1550 digitizer (Molecular Devices, CA, USA).

**Fluorescence in situ hybridization (FISH).** Mouse brains were extracted after anesthesia (Zoletil, 30mg/kg, i.p). These mouse brains were cut into areas containing the somatosensory cortex regions, and the sections were dropped quickly into liquid N2 for instant freezing. The frozen brain tissue was embedded in an optimal cutting temperature (OCT) compound block and kept overnight at -80°C in a deep freezer. The following day, the OCT block was transferred to a -20°C freezer for 2 hours and cryosectioned into 10-μm thick sections. FISH was performed using an RNAscope® Multiplex Fluorescent Reagent Kit (Advanced Cell Diagnostics, CA, USA) according to the manufacturer’s protocol. We used the probes designed by the manufacturer, including the RNAscope® Probe-Mm-Sst, Mm-Vip, Mm-Npy, Mm-Nos1, Mm-Gad1 (Advanced Cell Diagnostics, CA, USA). Confocal imaging was taken using a Leica TCS SP8 confocal microscope (Leica, Wetzlar, Germany) using a 20X objective lens. Quantification was performed with ImageJ software.
**Immunohistochemistry.** Mice were perfused through the heart with phosphate buffered saline (PH 7.4, PBS), followed by 4% paraformaldehyde (PFA). Brains were removed from the skull, post-fixed with 4% PFA for 12 hours at 4°C and then placed in a 30% sucrose solution at 4°C for 3 days. Using a cryomicrotome (CM 1950, Leica, Wetzlar, Germany), 40-μm thick frozen coronal sections were collected in 0.1M PBS. Sections were incubated in -20°C methanol for 10 min, washed in PBS, and incubated in a blocking solution (10% donkey serum in universal blocking solution, 00-8120 invitrogen, CA, USA) for 1h at room temperature. Next, sections were incubated in primary antibodies in PBS overnight at 4°C and washed in PBS 3 × 5 min. Then the sections were incubated in secondary antibodies for 2h at room temperature and washed in PBS 3 × 5 min. Nuclear counterstaining was performed with 100 ng/ml 4,6-diamidino-2-phenylindole (DAPI) solution (1:10000) in PBS for 10 min. Primary antibodies were used at the following concentration: rabbit anti-nNOS (1:800, Millipore, MA, USA), mouse anti-GAD67 (1:300, Millipore, MA, USA). Secondary antibodies conjugated with Alexa488 (1:350, Invitrogen, CA, USA) and Alexa568 (1:350, Invitrogen, CA, USA) were used to visualize the signals. Fluorescent images for cell counting were taken using a Leica TCS SP8 confocal microscope (Leica, Wetzlar, Germany) using 20X objective lens. Quantification was performed with ImageJ and Imaris software (Bitplane, UK, RRID:SCR_007370) software.

**Pharmacological agents.** 9,11-dideoxy-9a,11a-methanoepoxyprostaglandin F_2α_ (U46619, No.16450) was purchased from Cayman chemical (MI, USA). Tetrodotoxin (TTX, ab120055) was purchased from Abcam (Cambridge, UK). N-Methyl-D-aspartic acid (NMDA, M3262), AMPA hydrobromide (A9111), Muscimol (M1523), Baclofen (B5399), NBQX disodium salt
hydrate (N183), Somatostatin (S1763), and BIBP 3226 (B174) were purchased from Sigma-Aldrich (MO, USA). CGP 55845 hydrochloride (No.1248), Bicuculline methiodide (No.2503), D-AP5 (No.0106), and \(N^\omega\)-Propyl-L-arginine hydrochloride (L-NPA, No.1200) were purchased from Tocris Bioscience (Bristol, UK).

**Statistical analyses.** All reported values are presented as mean ± standard error of the mean (SEM). All statistical analyses were performed using the IBM SPSS statistical software (SPSS Inc., IL, USA, RRID:002865). Shapiro-Wilk tests were performed on all data sets to determine whether the data were normally distributed. Depending on the normality outcome, two-tailed independent samples t-tests or two-tailed Mann-Whitney U-tests were used for comparison of the two groups. For data sets which compared more than two groups, the Kruskal-Wallis test or one-way ANOVAs with Bonferroni post-hoc comparisons were used. The value of \(p < 0.05\) was accepted as statistically significant.

**Results**

**Validation of the mouse chronic stress model.**

Chronic restraint stress is a well-established protocol that induces anxiety-like behaviors in mice (Buynitsky and Mostofsky, 2009; Lee et al., 2015; Lee et al., 2018). The elevated plus maze (EPM), which contains both closed and open arms, is used to test the level of anxiety-like behaviors in animal subjects: the longer the time spent in the closed arms, the higher the level of anxiety-like behavior. In this study, we measured the duration of time both
control and stressed mice spent in the open and closed arms of the EPM during a 5-minute period. Chronically-stressed mice exhibited a higher level of anxiety-like behavior compared to the control mice (stressed vs. control; time spent in the closed arms: $171.40 \pm 2.77$ s vs. $122.29 \pm 4.74$ s, $U(123, 117) = 3042.5$, $p < 0.001$; open arms: $85.77 \pm 2.67$ s vs. $133.41 \pm 5.08$ s, $U(123, 117) = 3176.5$, $p < 0.001$, U-test; Figs. 1A and 1B). We tested 186 stressed mice with EPM, and 63 mice (~34% of stressed mice) showed stress resilience in our behavioral phenotyping results. This result is consistent with a previous study reporting 30–40% of chronically-stressed mice show stress-resilient phenotype (Golden et al., 2011). We measured plasma corticosterone in blood samples taken from control and chronically-stressed mice. Baseline corticosterone concentrations were elevated in the stressed group (stressed vs. control: $123.31 \pm 24.66$ ng/ml vs. $67.03 \pm 9.18$ ng/ml, $U(25, 25) = 166$, $p = 0.004$, U-test; Fig. 1C) indicating that HPA axis regulation was disrupted in those mice.

**Reduced hemodynamic response to forelimb stimulation in chronically stressed mice.**

To assess hemodynamic response to sensory stimulation in the chronically-stressed mice, we utilized optical intrinsic signal (OIS) imaging. Due to the well-organized topographical representation of the body surface in the primary somatosensory cortex, sensory-evoked neurovascular response is widely studied with OIS and vascular imaging (Berwick et al., 2008; Devor et al., 2008; Hillman, 2014; Iordanova et al., 2015; Cai et al., 2018). Thus, to study hemodynamic response, forelimb stimulation was recorded by OIS imaging of the forelimb activation region (S1FL) of the primary somatosensory cortex (Fig. 2A).
Since the stimulation-induced increase in Hbt results in a reduction of the reflected 546-nm OIS intensity (increase in absorption), Hbt-weighted OIS images taken during forelimb stimulation are presented as inverted values of relative change (Fig. 2B). Higher changes (red color) indicate larger increases in local cerebral blood volume (CBV) under the assumption that hemoglobin concentration remains constant. Overall, chronically-stressed mice showed significantly reduced stimulus-evoked changes in Hbt. First, peak Hbt response was significantly lower in the chronically-stressed group (stressed vs. control: 0.78 ± 0.09 % vs. 1.23 ± 0.08 %, \( U_{(10, 10)} = 16, p = 0.010 \), U-test; Figs. 2C and 2D). Second, the spatial extent of the stimulus-evoked Hbt response was smaller in the stressed animals (stressed vs. control: 0.66 ± 0.12 mm\(^2\) vs. 1.08 ± 0.16 mm\(^2\), \( t(18) = 2.109, p = 0.049 \), t-test; Fig. 2E). Additionally, the onset of the Hbt response tended to be delayed in the stressed group (stressed vs. control: 0.83 ± 0.11 s vs. 0.61 ± 0.09 s, \( t(18) = 0.841, p = 0.146 \), t-test; Fig. 2F), and the time to half-maximum of the peak was slightly less in the stressed animals (stressed vs. control: 1.79 ± 0.18 s vs. 2.12 ± 0.18 s, \( t(18) = 1.282, p = 0.216 \), t-test; Fig. 2G), however, neither difference was statistically significant.

Cardiovascular physiological parameters were measured throughout these experiments, since the altered hemodynamic responses in the stressed group may be due to changes in cardiovascular physiology. Both control and chronically-stressed mice maintained a normal range of physiological parameters under urethane anesthesia. There was no stimulation-induced change in heart rate (HR) and no significant difference between the two groups (stressed vs. control: 515.71 ± 30.27 bpm vs. 571.32 ± 20.36 bpm, \( t(18) = 1.525, p = 0.145 \), t-test; Figs. 2H and 2I). In addition, there was no difference in mean blood pressure (stressed vs. control: 79.2 ± 2.71 mmHg vs. 78.47 ± 0.49 mmHg, \( U_{(3, 3)} = 3, p = 0.513 \), U-test;
It is evident that chronic stress does not alter cardiovascular effects under our experimental conditions.

Summarily, chronic restraint stress decreased hemodynamic response to forelimb stimulation. Moreover, these results obtained with a mouse model are consistent with our previous work using a rat model of chronic restraint stress, which also showed reduced hemodynamic response to sensory stimuli (Lee et al., 2015).

**Neural response to forelimb stimulation was attenuated in chronically-stressed conditions.**

Simultaneously with OIS imaging, we also measured local field potential (LFP) in the somatosensory cortex to investigate whether reduced hemodynamic response to stimuli is associated with any changes in evoked neural activity (Fig. 2). LFP in the forelimb region was measured at a depth of ~300 μm, targeting layer II/III of the somatosensory cortex. Prior to insertion of the LFP recording electrode, a preliminary forelimb stimulation was performed, and the recording site was selected to be the center of the stimulus-evoked hemodynamic response, avoiding large surface vessels (Fig. 2A). LFP traces in Fig. 3A represent the average of 18 trials recorded during stimulation of one control and one stressed animal, respectively.

A comparison of evoked neural response amplitude revealed that stressed mice showed a markedly reduced relative increase of LFP power during the 20-second stimulation period (Fig. 3B). In other words, evoked neural responses were significantly suppressed in the chronically-stressed animals (stressed vs. control: $72.70 \pm 23.85 \%$ vs. $140.22 \pm 28.81 \%$).
neural activities were not significantly different (Figs. 3C and 3D). A power spectrum analysis of the stimulus-evoked LFP signal revealed significant suppression in the higher frequency bands (stressed vs. control; β band: 152.81 ± 48.20 % vs. 512.55 ± 111.77 %, \( U_{(10,10)} = 12, p = 0.004, U\)-test; γ: 137.65 ± 39.96 % vs. 572.98 ± 119.22 %, \( t(10.997) = 3.462, p = 0.005, t\)-test), whereas the power in the lower frequency bands remained unchanged (Figs. 3C and 3E). In light of these results, it is evident that reduced hemodynamic response to forelimb stimulus is likely to be associated with the attenuation of evoked neural activity in higher frequency bands.

**Chronic stress-induced alteration in the neurovascular coupling relationship.**

Although both neural and hemodynamic responses to forelimb stimuli are reduced in chronically-stressed conditions, there is a possibility that the neurovascular coupling relationship itself is unaffected. Therefore, we assessed whether the neurovascular coupling relationship had been altered by calculating the ratio of the evoked hemodynamic response to the evoked neural response. The calculation of the ratio between evoked neural activity and subsequent hemodynamic response was based on studies that assessed neurovascular coupling relationships in the cortical regions. The broad consensus from those studies is that there is a positive linear relationship between ongoing neural activity and the subsequent hemodynamic responses in the normal brain (Niessing et al., 2005; Devonshire et al., 2012; Iordanova et al., 2015; Lecrux et al., 2017). In chronically-stressed mice, the ratio between the peak value of Hbt changes (Fig. 2D) and the evoked neural activity (Fig. 3E) was
significantly changed in the beta and gamma frequency bands as well as over the total range of frequencies (2 to 100 Hz) (stressed vs. control; β band: 0.51 ± 0.07 vs. 0.21 ± 0.03, t(12.335) = -3.862, p = 0.002, t-test; γ band: 0.52 ± 0.07 vs. 0.19 ± 0.03, t(11.857) = -4.413, p = 0.0009, t-test; total neural activity: 0.66 ± 0.06 vs. 0.48 ± 0.05, U(10, 10) = 19, p = 0.019, U-test; Fig. 3F). In summation, chronic stress altered the neurovascular coupling relationship during functional hyperemia.

Vascular dynamics triggered by neuronal activation were altered in acute brain slices of chronically-stressed mice.

From in vivo data, we hypothesized that altered neurovascular coupling may have been caused by the dysfunction of specific cellular signaling that controls vascular responses. To elucidate which cell signals were primarily affected, we utilized acute brain slices, which allow better accessibility to measure complex signaling between neurons and blood vessels. During all vascular imaging experiments using the acute brain slices, we added the arteriole pre-constrictor, U46619 (65-100nM, Cayman Chemical, USA), to the bath to induce vascular tone (Cauli et al., 2004; Gordon et al., 2008; Longden et al., 2014; Institoris et al., 2015).

Within each slice, we selected 8-14μm diameter penetrating arterioles with thick smooth muscle in layer II/III of the somatosensory cortex.

First of all, we tested whether chronic stress alters vessel response to electrical stimulation. A concentric bipolar electrode was positioned 300μm away from the arteriole of interest to evoke neuronal excitation (Institoris et al., 2015). Then, we recorded the responses of the arteriole following 20Hz electrical stimulation (300μs, 2V, 5s) (Fig. 4C). In each group,
both vasodilation and vasoconstriction were observed, however, vasoconstriction was more prevalent in chronically-stressed mice (maximal diameter change versus baseline: 0.92±0.02), while vasodilation was observed more often in the control mice (1.08±0.02, $U_{(11,10)} = 2$, $p = 0.00019$, U-test; Fig. 4D). Second, the same electrical stimulation experiments were repeated, but with the addition of 1 μM tetrodotoxin (TTX). Use of this neurotoxin allowed us to determine whether these vascular responses were evoked by direct electrical stimulation to vessel or by neural activation. In the presence of TTX, there was almost no vascular response following 20Hz electrical stimulation in control mice (TTX: 0.98±0.01; Figs. 4E-F). These results indicate that the differences in vascular responses between normal and stressed mice are a consequence of differences in neuronal activity.

After confirming that the altered vascular responses observed in chronically-stressed mice was mediated by neuronal activity, we used various receptor agonists and antagonists to explore whether these neuronal changes originate from either the glutamatergic or GABAergic pathways. We then assessed the implication of NMDA receptors, which elicit the vasodilatory pathway via activation of the COX-2 enzyme (Gordon et al., 2008; Lecrux and Hamel, 2011; Lacroix et al., 2015). 30 μM NMDA application elicited arteriole vasodilation to the same degree in both stressed and control mice (stressed vs. control mice: 1.11±0.03 vs. 1.16±0.03, $U_{(7,9)} = 21$, $p = 0.266$, U-test; Figs. 4G-H). We, also, tested the implication of AMPA receptors, which has also been suggested to be a mediator of cerebral vasodilation via activation of a subsequent adenosine pathway (Ohata et al., 2006). 10 μM AMPA application did not evoke vasodilation in either group (stressed vs. control mice: 0.99±0.01 vs. 0.97±0.01, $U_{(5,5)} = 7$, $p = 0.251$, U-test; Figs. 4I-J). After confirming that NMDA-mediated vasodilation remain undamaged under chronically-stressed conditions, we tested a GABAergic pathway of
vascular response via GABA receptor agonists. GABA receptor agonist (100μM Muscimol+50μM Baclofen) stimulation induced dramatically dissimilar responses in control and stressed mice. While considerable arteriolar vasoconstriction occurred in control mice (0.95±0.02), some vasodilation was observed instead in chronically-stressed mice (1.03±0.01, $U_{13,11} = 20$, $p = 0.003$, U-test; Figs. 4K-L). There is also a possibility of a direct effect of GABA receptors on blood vessels. However, TTX abolished GABA agonist-mediated constriction, GABA receptor agonists did not act directly on blood vessels (stressed vs. control mice: 1.05±0.02 vs. 1.04±0.01, $t (12.06) = -0.33$, $p = 0.75$, t-test; Figs 4M-N). Consequently, NMDA-mediated vascular response is intact under chronic stress, while GABA-mediated vascular response is altered under chronic stress.

**Diminished frequency of inhibitory postsynaptic currents (IPSCs) from pyramidal cells in chronically-stressed mice.**

Examining the electrophysiological properties of pyramidal cells through whole-cell patch clamp recording allowed us to confirm whether synaptic transmission was altered under chronic stress conditions. Measurements of spontaneous excitatory postsynaptic currents (sEPSCs) from neurons in the somatosensory cortex showed that sEPSC amplitude (stressed vs. control mice: 12.12±1.24 vs. 9.85±0.74 pA, $U_{11,10} = 34$, $p = 0.139$, U-test) and frequency (4.90±0.97 vs. 3.03±0.53 Hz, $t (19) = -1.649$, $p = 0.116$, t-test) had an increasing trend, albeit not significantly different, between the two groups (Figs. 5B and 5D-E). However, the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) (stressed vs. control mice: 2.88±0.30 vs. 3.85±0.32 Hz, $t (21) = 2.204$, $p = 0.039$, t-test) was significantly
reduced in chronically-stressed mice with the sIPSC amplitude remaining unchanged (27.74±1.62 vs. 26.43±2.06 pA, t (21) = -0.508, p = 0.617, t-test; Figs. 5C and 5D-E).

Furthermore, we measured tonic GABA currents that are mainly mediated by the extrasynaptic GABA_A receptor, whose activity has bidirectional effects on hyperemia (Jessen et al., 2015). The amplitude of tonic GABA currents (stressed vs. control mice: 4.94±2.14 vs. 1.58±0.59 pA, U(12,10) = 47.5, p = 0.405, U-test) was not notably different between the two groups (Figs. 5F-H). Collectively, these observations suggest that chronic stress disrupts pre-synaptic GABA release evoked by neural activity and changes the balance of excitatory and inhibitory currents (E/I balance).

**Dysfunction of GABA_A receptors contributes to neurovascular coupling impairment in chronically-stressed mice.**

GABA, which is a major inhibitory neurotransmitter, is modulated via GABA_A and GABA_B receptors. GABA_A is a ligand-gated ion receptor with a chloride ion-selective pore while GABA_B is a G protein-coupled receptor. Each GABA receptor type has been reported to affect neurovascular coupling (Fergus and Lee, 1997; Jessen et al., 2015). We differentiated between the two GABA receptors’ impact on neurovascular coupling in chronically-stressed mice by using either a GABA_A receptor antagonist (bicuculline, 100 μM) or a GABA_B receptor antagonist (CGP 55845, 20 μM). Each antagonist was applied for the 20 minutes preceding 20Hz focal electrical stimulation and maintained throughout the experiment. In control mice, selective GABA_A receptor blockade via bicuculline induced vasoconstriction following electrical stimulation (ACSF: 1.08±0.01, Bicuculline: 0.88±0.22,
p<0.001; Figs. 6A and 6E), whereas selective GABA\textsubscript{B} receptor blockade with CGP 55845 produced no significantly different responses in vascular dynamics (CGP 55845: 1.09±0.01, p=1; Figs. 6C and 6G). To determine whether the vascular responses caused by bicuculline in the control group was independent from glutamatergic signaling, we performed the same bicuculline experiment while blocking NMDA and AMPA receptors. Since the experimental results under receptor blockade did not prove to differ, it is apparent that vascular responses triggered by bicuculline is likely to be independent to glutamatergic signaling (Bicuculline with AP5 and NBQX: 0.92±0.04, $F_{(3,26)} = 26.708$, p<0.001, one-way ANOVA with Bonferroni post-hoc test; Figs. 6B and 6F). In stressed mice, similar vascular responses were shown regardless of drug application contrasting with the results obtained from the controls (ACSF: 0.94±0.02, Bicuculline: 0.91±0.02, p=1, Bicuculline with AP5 and NBQX: 0.94±0.04, p=1, CGP 55845: 0.95±0.03, $F_{(3,27)} = 0.386$, p=1, one-way ANOVA with Bonferroni post-hoc test; Figs. 6B and 6D). Ultimately, our observations corroborate the prominence of the role of GABA\textsubscript{A} receptor metabolites in maintaining normal neurovascular coupling.

Impaired neuronal NOS-mediated neurovascular coupling in chronically-stressed mice.

Different subtypes of GABAergic interneurons control vasodilation by releasing nitric oxide (NO) and vasoactive intestinal peptide (VIP). Some subtypes also control vasoconstriction by releasing the neuropeptides, such as somatostatin (SOM) and neuropeptide Y (NPY) (Cauli et al., 2004; Uhlírova et al., 2016). To evaluate which GABAergic interneurons create dysfunctional hemodynamics in chronically-stressed animals,
we investigated neurovascular coupling under specific GABAergic interneurons’ subtype agonists and antagonists. Specific GABAergic interneurons’ subtype agonists and antagonists were applied for the 20 minutes preceding 20Hz focal electrical stimulation and maintained throughout the experiment. When the drug, somatostatin, was applied, the vascular response to local electrical stimulus was not altered compared with ACSF conditions in either the control (ACSF: 1.08±0.02, SOM: 1.04±0.02, p = 0.981; Fig. 7A) or the stressed group (ACSF: 0.93±0.02, SOM: 0.92±0.01; Fig. 7E). Thus, SOM-expressing interneurons have less relevance to alteration of NVC evoked by chronic stressors. Like somatostatin, selective NPY Y1 receptor blockade with 1 μM BIBP 3226 also had little effect on vascular response compared with the ACSF condition in either the control group (BIBP 3226: 1.07±0.01, p=1; Fig. 7B) or the stressed group (BIBP 3226: 0.97±0.02; Fig. 7F). In contrast, vasoconstriction was strongly induced in the control group under 10 μM L-NPA, which is a selective neuronal nitric oxide synthase (nNOS) receptor blocker (L-NPA: 0.95±0.03, F(3,35) = 5.952, p=0.002, one-way ANOVA with Bonferroni post-hoc test; Fig. 7C). nNOS is one of the isomers of the enzyme involved in the synthesis of nitric oxide (NO) which is a major vasodilator that regulates cerebral blood flow (Cauli and Hamel, 2010; Kilduff et al., 2011). Unlike the control group, the stressed mice showed similar vascular response even in the presence of L-NPA (L-NPA: 0.98±0.02, χ²(3) = 5.432, p= 0.143, Kruskal-Wallis test; Fig. 7G) Therefore, the nNOS-mediated vasodilation pathway is likely to be impaired by chronic stress.

Reduced numbers of nNOS-expressing GABAergic interneurons in chronically-stressed mice.

After confirming the importance of the role of nNOS-expressing neurons in
neurovascular coupling, we checked the mRNA expression of several types of GABAergic interneurons in both control and chronically-stressed mice by utilizing fluorescence in situ hybridization (FISH). Only the mRNA expression of nNOS was significantly reduced in chronically-stressed mice (stressed vs. control mice: 379.56±95.65 vs. 815.37±137.59 cells/mm², $U(5,5) = 3$, $p=0.047$, U-test; Figs. 8D and 8H). By comparison, the mRNA expression of SOM (stressed vs. control mice: 8388.89±645.69 vs. 6791.69±465.60 cells/mm², $U(5,7) = 8$, $p=0.123$, U-test), VIP (4540.36±522.48 vs. 3316.69±429.27 cells/mm², $U(5,7) = 6$, $p=0.062$, U-test) and NPY (7207.65±946.98 vs. 6110.79±907.24 cells/mm², $U(5,7) = 12$, $p=0.372$, U-test) appeared to have no differences between the two groups (Fig. 8).

There are two classes of nNOS-expressing neurons; type 1 and type 2. Type 1 nNOS neurons show large somata with strong IHC intensity while type 2 nNOS neurons have small somata and weak intensity (Yan and Garey, 1997; Perrenoud et al., 2012). Type 1 nNOS neurons are predominantly located in deeper layers of the cortex and co-express SOM, NPY and PV. Conversely, type 2 nNOS neurons are mainly located in layer II/III and VI and co-express PV, SOM and VIP (Magno et al., 2012; Perrenoud et al., 2012). Hence, we further confirmed the expression of each type of nNOS protein level using IHC. As a result, type 1 nNOS neurons were considerably reduced in chronically-stressed mice (stressed vs. control mice: 372.08±29.39 vs. 492.11±33.50 cells/mm³, $U(11,12) = 27$, $p=0.016$, U-test; Fig 9B). Differences in type 2 nNOS neurons, while statistically insignificant, were apparently lower in chronically-stressed mice (671.95±101.13 vs. 903.50±91.83 cells/mm³, $U(6,6) = 7$, $p=0.078$, U-test; Fig 9D), thus it is difficult to entirely rule out the possibility that type 2 nNOS neurons are unaffected by chronic stress. These results suggest that type 1 nNOS neurons have more vulnerability to chronic stressor than type 2 nNOS neurons. Collectively, our
experimental outcomes imply that the reduction of nNOS expression contributes to the nNOS-mediated neurovascular coupling impairment found in chronically stressed mice. nNOS-expressing neurons also co-express SOM and other neuropeptides such as NPY, which are known to be vasoconstrictors. Indeed, SOM seems to induce slight vasoconstriction trends in control mice and NPY receptor blockade also seems to induce slight vasodilation trends in stressed mice (Fig. 7). Thus, future experiments using cell type specific modulation by optogenetic or chemogenetic technique would be required to address that the exact role of GABAergic interneurons in controlling cerebral blood flow in case they co-express neuropeptides known as different vasomodulatory action.

Discussion

This study demonstrates that neurovascular coupling is disrupted during functional hyperemia in chronically-stressed animals. The purpose of this study was to investigate both in vivo and ex vivo the underlying cellular mechanisms that drive such alterations. We compared sensory stimulus-evoked hemodynamic and neural responses in unstressed and stressed mice. These comparisons revealed an altered relationship between gamma frequency activity and hemodynamic response during forelimb stimulation in chronically-stressed animals. Utilizing ex vivo vascular imaging along with pharmacological tests, patch-clamp recording and FISH techniques, several key results emerged. First, there was a decrease in sIPSC frequency in chronically-stressed mice. Second, GABA_A receptor and nNOS-mediated vascular responses are altered by chronic stress. Third, a reduced number of nNOS-expressing cells was observed in chronically-stressed animals. Overall, our study clearly reveals that chronic stress has highly negative impacts on neurovascular coupling, disrupting
Neuronal origin of altered NVC under chronically-stressed condition.

In our in vivo data, chronic stress was associated with both attenuated hemodynamic response and LFP response to forelimb stimulus. Logothetis et al. (Logothetis, 2008) described the alteration in hemodynamic signal when the excitation-inhibition balance (E/I balance) changes. Changes in neural activity while maintaining the E/I balance would lead to proportional changes in hemodynamic response. Adopting this idea, if the chronic stress-associated reduction in hemodynamic response is directly proportional to the attenuated neural response, the reduced overall power of neural activity would be likely responsible for the decreased hemodynamic response. Results in Fig. 3F, however, show that the NVC relationship was changed in chronically-stressed mice. Thus, our interpretation of these data is that an imbalance between the excitatory and inhibitory cortical microcircuits is associated with chronic stress, and there is likely to be an additional component other than the reduction in net excitation during sensory stimulation to account for the reduction in hemodynamic response.

The results of our patch-clamp and LFP recordings suggest that the additional component that contributes to the altered NVC relationship may be dysfunctional inhibitory neuronal activity. Reduction in sIPSC frequency in the stressed brain (Fig. 5E) implies that GABA release from pre-synapses is decreased under chronic stress. Previous reports showed similar outcomes in terms of GABAergic interneuron activity. Under the chronically-stressed condition, GABAergic inhibition was attenuated and the number of GABAergic interneurons...
was reduced (Czéh et al., 2015; Csabai et al., 2018; Czéh et al., 2018). Since inhibitory neural circuits in the neocortex precisely control the E/I balance (Tremblay et al., 2016), change in sIPSC can modulate the E/I balance. Since a balanced interaction between excitation and inhibition generates gamma oscillation (Buhl et al., 1998), a modulation in the E/I balance can be reflected as altered gamma activity (Levin and Nelson, 2015). Here, we observed a selective reduction in the evoked gamma activity under chronic stress (Fig. 3E). Reports that emphasize the role of GABAergic interneurons on generating gamma oscillations (Cardin et al., 2009) also support that chronic stress exposure may differently affect excitatory and inhibitory cortical networks. Since evoked gamma activity during functional hyperemia correlates better with hemodynamic signal in the neocortex compared to lower frequency activity (Niessing et al., 2005), selective attenuation in gamma activity may contribute to the decrease in hemodynamic response. Together, chronic stress induces an E/I imbalance in cortical microcircuits by affecting inhibitory neuronal activity, which may lead to reduction in gamma activity and hemodynamic response. These results suggest that the alteration of NVC under chronic stress originates from neuronal alteration.

Contribution of glutamatergic neurons for altered NVC in chronically-stressed mice.

The activation of NMDA receptors in cortical excitatory neurons release COX-2 products, which is followed by hemodynamic response (Gordon et al., 2008; Lecrux and Hamel, 2011; Lacroix et al., 2015). Hence, we sought to determine the implications of excitatory neuron activity on vascular response using acute brain slices. In this study, NMDA application induced vasodilation, consistent with a prior study (Lacroix et al., 2015), but a
similar degree of vasodilation was shown in both control and stressed mice. This suggests that NMDA-mediated vascular responses remain intact under chronically-stressed conditions. Metabotropic glutamate receptors (mGluRs) are expressed in both reactive glia and neuronal cells, and controversy remains regarding their role in functional hyperemia. It has been suggested that the blockade of group I mGluRs reduce evoked CBF responses to whisker stimulation (Lecrux et al., 2011). However, another group has shown that the blockade of mGluR5 had no impact on evoked hemodynamic changes (Calcinaighi et al., 2011). Consequently, definitive evidence on the mGluRs’ role in functional hyperemia is still lacking and remains to be determined.

**Role of each GABA receptor on altered NVC in chronically-stressed mice.**

Evoked CBF response to sensory stimulation recruits cortical excitatory neurons and inhibitory neurons, and, interestingly, GABA interneurons account for a large portion of c-Fos-positive neurons following sensory stimulation (Lecrux et al., 2011). Prior studies showing that optogenetic stimulation of vesicular GABA transporter (VGAT) evoked cerebral blood flow increases (Anenberg et al., 2015; Vazquez et al., 2018) also support the crucial role of GABAergic interneurons on NVC. In the present study, we also observed that GABA receptor agonists induced significant differences between the control and stressed groups, indicating a strong correlation between chronic stressors and GABA-mediated vascular response. The vasoconstriction response following GABA agonist treatment was observed in the control group, which is consistent with prior demonstrations that high concentrations of the GABA<sub>A</sub> receptor agonist muscimol attenuates CBF changes (Mathiesen
et al., 2011) and GABA$_B$ receptor agonists induce vasoconstriction in the hippocampus in vitro (Fergus and Lee, 1997). To more fully explore the specific role of each GABA receptor on vascular response, we applied blockers of GABA$_A$ and GABA$_B$ receptors, separately. Our data showed that the blockade of GABA$_A$ receptors totally altered vascular response derived from electrical stimulation. These data suggesting the involvement of GABA$_A$ receptor on functional hyperemia are consistent with recent observations showing that GABA$_A$ receptor antagonism reduced CBF response to whisker or basal forebrain (BF) stimulation (Kocharyan et al., 2008; Lecrux et al., 2011). Collectively, our findings suggest that GABA$_A$ receptor-mediated vascular response is affected by chronic stress.

Contribution of nNOS-expressing neurons on altered NVC in chronically-stressed mice.

There are highly diverse populations of GABAergic interneurons in the neocortex (Rudy et al., 2011; Kepecs and Fishell, 2014) and VIP-, SOM-, NOS-, ChAT- and PV-expressing GABA interneurons are selectively recruited by different afferent inputs (Lecrux and Hamel, 2016). They have been implicated in bidirectional effects dependent on their vasoactive messengers, eg. vasodilation derived by VIP/NOS and vasoconstriction derived by SOM/NPY (Cauli et al., 2004; Uhlirova et al., 2016). In this study, we demonstrated that only in the presence of nNOS inhibitors does the vasodilation response of the control group convert to vasoconstriction, thereby mimicking the stressed group. This is consistent with previous studies showing that CBV and CBF responses evoked by photo-stimulation of VGAT was diminished under an NOS blocker (Vazquez et al., 2018), and functional hemodynamic changes elicited by sensory stimulation were significantly reduced under
nNOS specific blockers or in nNOS knock out mice (Kitaura et al., 2007; Stefanovic et al., 2007). In addition, we confirmed that chronically-stressed mice exhibit a reduction in nNOS-expressing neurons via histological approaches matching the results of our previous study reporting that nNOS expression is decreased in chronically-stressed rats using western blotting (Lee et al., 2015). To sum up, our findings strongly support that NOS interneurons have important role in maintaining NVC.

Limitations and future perspectives

The current study has several limitations that should be addressed in future research. One issue deals with the use of anesthetics. We used anesthesia to avoid acute stress effects coming from handling and head fixation. Though urethane anesthesia is known to preserve a stable state of neurovascular coupling (Sceniak and MacIver, 2006; Berwick et al., 2008; Boorman et al., 2015), we note that neurovascular properties in the awake state can be different in some aspects as suggested by other reports (Martin et al., 2013; Gao et al., 2017). Thus, chronic stress effects on sensory-evoked neurovascular responses in the awake state should be further investigated. Another is that our investigation of the chronic stress effects on nNOS-mediated vascular responses were done only in the ex vivo situation. Whether the results would be reproduced in vivo during sensory stimulation remains to be addressed. To answer this question, comparing the cortical hemodynamics in the unstressed and stressed animals while modulating nNOS activity with nNOS antagonists would be required. This would lead to a better understanding of chronic stress-induced alteration in NO signaling pathways. Moreover, nNOS neurons’ activity is highly related to sleep homeostasis
(Gerashchenko et al., 2008). Since nNOS-knockout mice show altered sleep homeostasis (Morairty et al., 2013), it would be very interesting to study sleep pattern changes in chronically-stressed mice.

**Conclusion**

In conclusion, our results suggest that chronic stress exposure impairs NVC by affecting the expression and function of nNOS-expressing GABAergic interneurons. Since chronic stress is a contributory factor for the progression of stress-related disorder, these findings may suggest an important role of nNOS signaling pathways in preventing or treating stress-related disorders.

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**Figure Legends**

**Figure 1.** Behavioral and physiological validation of the chronic stress mouse model. (A) Representative heatmaps showing the exploration of mice during a 5-minute EPM test. Each image was obtained from a single mouse. Red color indicates more time spent in an area, with the blue color indicating the opposite. (B) Average duration of time spent in each area of the EPM (control, n = 117; stressed, n = 123). (C) Comparison of baseline plasma corticosterone concentrations (control, n = 25; stressed, n = 25). All values are mean ± SEM. ††: p < 0.01, †††: p < 0.001, Mann-Whitney U-test.

**Figure 2.** Reduced hemodynamic responses to forelimb stimulus in chronically-stressed animals. (A) Experimental settings for *in vivo* OIS imaging. Light reflected from the exposed S1FL cortex (i.e., the forelimb region of the primary somatosensory cortex) was filtered at the 546 nm wavelength, which is a measure of the total hemoglobin (Hbt). To assess the neurovascular coupling relationship during functional hyperemia, local field potentials (LFP) were measured simultaneously with the OIS imaging. (B) Representative Hbt-weighted OIS images during forelimb stimulation in control (left) and stressed mice (right). Each image represents the average of 18 trials from a single individual. Scale bars: 1 mm. (C) Time-course traces of relative changes in the evoked Hbt signal following forelimb stimulation, relative to baseline levels (control, black, n=10; stressed, red, n=10). (D) Average of the maximum evoked Hbt change. (E) Comparison of the area of activated region (spatial extent) for the time point at which the maximum Hbt change was observed. (F) Time of onset, and
(G) Time to reach half-maximum of the first peak of the Hbt response. (H) Time-course traces of heart rate monitored during OIS imaging sessions including the sensory stimulation period (indicated with the yellow box). (I) Average of the heart rate measured throughout the experiments (control, n = 10; stressed, n = 10). (J) Blood pressure measured in urethane-anesthetized mice (control, n = 3; stressed, n = 3) using a tail-cuff blood pressure measurement system. All values are mean ± SEM. †: p<0.05, Mann-Whitney U-test. *: p<0.05, independent t-test. n.s.: no significance.

**Figure 3.** Reduced neural response to forelimb stimulus and alteration of the neurovascular coupling relationship in chronically-stressed animals. (A) Representative raw LFP traces acquired from control and stressed mice. (B) Time-course traces of relative changes in the evoked neural activity following forelimb stimulation, relative to pre-stimulus baseline levels (control, black, n = 10; stressed, red, n = 10). (C) Power spectra of LFP recorded during stimulation (solid lines; control group in black and stressed group in red) and pre-stimulus baseline (dashed lines) periods. The grey-colored box indicates the frequency range (55 - 65Hz) excluded from all analyses due to use of a 60 Hz notch filter. (D) Comparison of baseline neural activity in different frequency ranges. (E) Comparison of the evoked neural activity in different frequency ranges. Values in (B-E) are mean ± SEM. (F) Box plot (box and whisker diagram) of the ratio of the evoked Hbt response to the evoked neural response. †: p < 0.05, ††: p < 0.01, Mann-Whitney U-test. **: p < 0.01, ***: p < 0.001, Independent samples t-test. n.s.: no significance.
Figure 4. Altered vascular dynamics in chronically-stressed mice, as measured in acute brain slices. (A) The schematic setup showing the experiment. (B) Representative images of baseline and post-electrical 20 Hz stimulation arterioles in the somatosensory cortex of control and stressed mice. Red vertical lines were traced along vascular walls for visualization of penetrating arterioles. (C-D) Time-course traces and maximal relative amplitudes of arteriolar diameter changes following electrical stimulation for control (black, n = 10) and stressed brain (red, n = 11). Clearly, stimulation induces dilation for the control, but constriction for the stressed brain slice. (E-F) Time-course traces and maximal relative amplitudes of vascular diameter changes after stimulation in the presence (green, n = 6) or absence of TTX (gray, n=5) in the control mouse brain slice. In the control brain slice, stimulation induces vascular dilation (1.08±0.01), which is abolished by TTX application (0.98±0.01, U(6,5) = 0, p=0.004, U-test). (G-J) Time-course traces and maximal relative amplitudes of vessel diameter change induced by 2 minutes’ application (horizontal black line) of 30μM NMDA (G, H; control n = 9, stressed n = 7) and 10μM AMPA (I, J; control n = 5, stressed n = 5) for control (black) and stressed brain slice (red). Both NMDA and AMPA mimic the action of glutamate. Neither compounds produce any significant difference in vascular diameter for control vs. stressed slice. (K-M) Time-course traces (K, M) and maximal relative amplitudes (L, N) of vessel diameter change induced by 2 minutes’ treatment of GABA receptor agonists (100μM Muscimol + 50μM Baclofen) in the absence (K, M) and presence of TTX (L, N) for control (black, n = 13 without and 8 with TTX) and stressed brain slices (red, n = 11 without and 11 with TTX). GABA agonists induce significantly larger increase in vascular diameter in stressed brain slice, but its effect is suppressed under TTX. All values are mean ± SEM. **: p<0.01, ***: p<0.001, ns: not
Figure 5. In chronically-stressed animals, significantly reduced the frequency of inhibitory postsynaptic currents (IPSCs) in pyramidal cells of the somatosensory cortex. (A) The schematics showing patch-clamp recording from pyramidal cells in somatosensory cortex. (B-C) Representative traces of excitatory postsynaptic currents (EPSCs) (B) and IPSCs (C) in control (black, top) and stressed mice (red, bottom). (D) The amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) (control: n=10, stressed: n=11) and spontaneous inhibitory postsynaptic currents (sIPSCs) (control: n=10, stressed: n=13) (E) The frequency of sEPSCs (control: n=10, stressed: n=11) and sIPSCs (control: n=10, stressed: n=13). (F,G) Representative traces of tonic GABA$_A$R currents in control (black, left) and stressed mice (red, right). (H) The amplitude of tonic GABA$_A$R currents measured in two groups (control: n=10, stressed: n=11) and the amplitude of tonic GABA$_A$R currents measured with treatment of 5 μM GABA in two groups (control: 13.28±2.26 pA, n=9, stressed: 12.3±2.13, n=10, U(9,10) = 38, p=0.567, U-test). All values are mean ± SEM. *: p<0.05, n.s.: not significant.

Figure 6. Alteration of GABA$_A$ receptor-mediated vascular responses induced by 20Hz focal electrical stimulation. (A-C, E-G) Time-course traces of arteriolar diameter change in control (A-C) and stressed brain tissues (E-G) exposed to two conditions: under artificial cerebrospinal fluid (ACSF) circulation (black or red) or with treatment of drug (blue). 100 μM bicuculline, a GABA$_A$ antagonist (A,E), 100 μM bicuculline with glutamergic antagonists (10 μM AP5 + 5 μM NBQX) (B,F), and 10 μM CGP 55845, a GABAB
antagonist (C,G) were used as drug circulation. (D) Maximum diameter change amplitude in the control group following each drug treatment (ACSF: n=10, Bicuculline: n=8, Bicuculline with AP5 and NBQX: n=5, CGP 55845: n=7). (H) Maximum diameter change amplitude in the chronically-stressed group following each drug treatment (ACSF: n=11, Bicuculline: n=8, Bicuculline with AP5 and NBQX: n=5, CGP 55845: n=7). All values are mean ± SEM. ***: p<0.001, ns: not significant, Statistical significance was tested with the one-way ANOVA followed by Bonferroni post-hoc tests according to the results of a normal distribution test (Shapiro-Wilk test).

Figure 7. Alteration of nNOS-mediated vascular responses induced by 20Hz focal electrical stimulation. (A-C, E-G) Time-course traces of arterial diameter change in control (A-C) and stressed brain tissues (E-G) under ACSF (black or red) or with drug treatment (green). Drug was 1 μM somatostatin (A,E), a neuropeptide that agonist for somatostatin, 1 μM BIBP 3226 (B,F), an antagonist for the neuropeptide Y receptor, and 10 μM L-NPA (C,G), a nNOS inhibitor. (D) Maximum diameter change amplitude following each drug treatment in the control group (ACSF: n=10, Somatostatin: n=13, BIBP 3226: n=6, L-NPA: n=10). (H) Maximum diameter change amplitude following each drug treatment in the chronically-stressed group (ACSF: n=11, Somatostatin: n=12, BIBP 3226: n=5, L-NPA: n=12). All values are mean ± SEM. **: p<0.01, ns: not significant. A one-way ANOVA followed by Bonferroni post-hoc tests was used for analysis of a drug treatment effect in the control group, and the Kruskal-Wallis Test was used for statistical analysis of the chronically-stressed group according to the results of a normal distribution test (Shapiro-Wilk test).
Figure 8. Decreased mRNA expression of nNOS in the somatosensory cortex of chronically-stressed mice. (A,E) Somatostatin (SOM) mRNA expression in the somatosensory cortex of both control and chronically-stressed mice (control: n=7, stressed: n=5). (B,F) Vasoactive intestinal peptide (VIP) mRNA expression (control: n=7, stressed: n=5). (C,G) Neuropeptide Y (NPY) mRNA expression (control: n=7, stressed: n=5). (D,H) Neuronal nitric oxide synthase (nNOS) mRNA expression (control: n=5, stressed: n=5). All values are mean ± SEM. *: p<0.05, ns: not significant, Mann-Whitney U test.

Figure 9. Decreased protein expression of nNOS-expressing neurons in the somatosensory cortex of chronically-stressed mice. (A,C) Cortical layer-dependent (left) and expanded IHC images (right) of nNOS-expressing neurons from control and chronically stressed mice. Type 1 nNOS-expressing neurons have large soma with strong IHC intensity, while type 2 nNOS neurons have small soma with weak intensity. Scale bars in sub-plots: 20 μm. (B, D) The number of type 1 (control: n=12, stressed: n=11) and type 2 nNOS neurons (control: n=6, stressed: n=6). All values are mean ± SEM. *: p<0.05, Mann-Whitney U test.
A) Change in diameter (normalized to baseline) with electrical stimulation.

B) Control and Stressed groups with baseline and 20Hz stimulation images.

C) Electrical stim with max. diameter change (normalized to baseline).

D) TTX with max. diameter change (normalized to baseline).

E) AMPA with max. diameter change (normalized to baseline).

F) NMDA with max. diameter change (normalized to baseline).

G) GABA agonists with max. diameter change (normalized to baseline).

H) GABA agonists with max. diameter change (normalized to baseline).

I) TTX with max. diameter change (normalized to baseline).

J) TTX with max. diameter change (normalized to baseline).

K) TTX with max. diameter change (normalized to baseline).

L) TTX with max. diameter change (normalized to baseline).

M) TTX with max. diameter change (normalized to baseline).

N) TTX with max. diameter change (normalized to baseline).
Table: Change in Diameter (normalized to baseline)

| Treatment | Time (s) | Change in Diameter (normalized to baseline) |
|-----------|---------|---------------------------------------------|
| Control   |         |                                             |
| Stressed  |         |                                             |

Graphs: Bicuculline (AP5+NBQX) and CGP 55845 effects on diameter change.
L-NPABIBP 3226

Change in diameter (normalized to baseline)

Time (s)

Change in diameter (normalized to baseline)

Time (s)

Change in diameter (normalized to baseline)

Time (s)

**n.s.**
A 1 2/3 4 5 6
100μm
100μm
nNOS1
GAD67
Merge
nNOS1
GAD67
Merge

0
*
Cells per mm
3

B nNOS type 1

600
500
400
300
200
100
0
Control Stressed

* p = 0.078

C Stressed

D nNOS type 2

1200
1000
800
600
400
200
0
Control Stressed

p = 0.078

nNOS type 1 B

Control Stressed

200
300
400
500
600

Cells per mm
3