Cocaine-induced ischemia in prefrontal cortex is associated with escalation of cocaine intake in rodents

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Abstract

Cocaine-induced vasoconstriction reduces blood flow, which can jeopardize neuronal function and in the prefrontal cortex (PFC) it may contribute to compulsive cocaine intake. Here we used integrated optical imaging in a rat self-administration and a mouse non-contingent model, to investigate whether changes in the cerebrovascular system in the PFC contribute to cocaine self-administration, and whether they recover with detoxification. In both animal models, cocaine induced severe vasoconstriction and marked reductions in cerebral blood flow (CBF) in the PFC, which were exacerbated with chronic exposure and with escalation of cocaine intake. Though there was a significant proliferation of blood vessels in areas of vasoconstriction (angiogenesis), CBF remained reduced even after one month of detoxification. Treatment with Nifedipine (Ca^{2+} antagonist and vasodilator) prevented cocaine-induced CBF decreases and neuronal Ca^{2+} changes in the PFC, and decreased cocaine intake and blocked reinstatement of drug seeking. These findings provide support for the hypothesis that cocaine-induced CBF reductions lead to neuronal deficits that contribute to hypofrontality and to compulsive-like cocaine intake in addiction, and document that these deficits persist at least one month after detoxification. Our preliminary data showed that nifedipine might be beneficial in preventing cocaine-induced vascular toxicity and in reducing cocaine intake and preventing relapse.

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Conflict of Interest
The authors declare no conflict of interest.

Introduction:

Deficits in the function of the prefrontal cortex (PFC) play a crucial role in promoting compulsive cocaine use in humans (Rapinesi et al., 2016). Prefrontal dysfunction is in part attributable to cocaine-induced disruption of dopamine striato-cortical circuits (Volkow et al., 2015). However, cocaine's direct disruption of cerebral blood vessels and cerebral blood flow (CBF) is also likely to contribute to and exacerbate hypofrontality. Indeed, cocaine abusers are at higher risk of ischemic and hemorrhagic strokes in the brain than non-abusers (Tuchman et al., 1987; Levine et al., 1987; Levine et al., 1991) and imaging studies in cocaine abusers have documented marked decreases in CBF, which are most prominent in the PFC (Volkow et al., 1988). Similarly, studies in rodents have shown that chronic cocaine triggers vasoconstriction, reduces CBF and results in cerebral ischemia (Zhang et al., 2016). However, the extent to which cocaine-induced CBF decreases affect neuronal activity in the PFC and contribute to compulsive-like cocaine taking and the extent to which the CBF decreases recover with detoxification have been minimally investigated (Zhang et al., 2016).

To investigate the changes in the cerebrovascular system with cocaine exposure and their rate of recovery with detoxification along with their relationship to compulsive cocaine intake we used an integrated optical imaging tool in rodents. We previously reported, using ultrahigh-resolution optical coherence Doppler tomography (μODT) for 3D imaging of CBF, that acute cocaine reduced cortical CBF and that repeated injections within the same session (2–3 doses) exacerbated these deficits triggering cortical microischemia in the mouse brain (Ren et al., 2012; You et al., 2014). However, such studies were done in the somatosensory cortex (not the PFC), cocaine was non-contingently administered (rather than self-administered) and it was given acutely (3 doses within 3 hrs). To address these limitations, here we performed two parallel studies that assessed the effects of chronic cocaine in neurovascular networks in the PFC: one in a rat model that allowed us to assess the effects of compulsive-like cocaine self-administration (long access, LgA) (Ahmed et al., 1998), and the other in a longitudinal mouse model that allowed us to monitor changes as a function of chronicity and withdrawal. The LgA model emulates the compulsive-like patterns of cocaine intake characteristic of cocaine addiction (Koob 2011; Koob 2009). In this model rats are allowed to intravenously self-administer cocaine with extended (long) access (LgA, 6hr/day, 0.5mg/kg/injection), which increases their motivation to take more cocaine leading to escalation in intake. The longitudinal mouse model uses an implanted cranial window on the PFC (also on the somatosensory cortex to serve as comparison), which allowed us to assess cocaine-induced changes as a function of chronicity and to assess if the changes recovered following withdrawal. Finally, we examined whether pre-treatment with nifedipine, a Ca^{2+} antagonist and vasodilator, could prevent cocaine-induced changes in CBF and reduce cocaine-self administration and prevent reinstatement of cocaine seeking.

We hypothesized that both cocaine self-administration and non-contingent cocaine administration would result in vasoconstriction, reductions in CBF, and ischemia in the PFC and that prevention of vasoconstriction and ischemia by nifedipine would reduce escalation of cocaine self-administration and prevent reinstatement of cocaine seeking. We also hypothesized that without treatment cocaine-induced reductions in CBF and neuronal activity would persist after detoxification.

Materials and Methods:

Animal preparation and physiology measurement

All procedures were carried out in accordance with animal protocols approved by the Institutional Animal Care and Use Committees of Stony Brook University. A total of 83 animals were used, including 53 rats and 30 mice, for the various experiments in this study (for detail, see Supplemental 1, Table S1). For all animals (rats or mice), cocaine pre-treatment was conducted while animals were awake. Imaging was conducted while animals were anesthetized with 1.5–2.5% isoflurane in a 60–70% O2/Air mixture either by intubation (rats) or face mask (mice).

For the rat study, we continuously monitored physiological measures during the imaging procedure using a Small Animal Instrument (Module 224002) to record heartbeat, mean arterial blood pressure (MABP), respiration rate and body temperature. The end-tidal CO2 was monitored continuously using a Poet IQ2, (Criticare Technologies). Rats were kept under stable conditions, including MABP, body temperature, respiration and heart rate. The end-tidal CO2 was kept in the normocapnic range (~ 35–45 mmHg, Supplementary 6, Table S2).

For the mouse study, heartbeat, respiration rate and body temperature were measured noninvasively with a Small Animal Instrument (Module 224002) during imaging. However, the small blood volume of mice precluded measurements of blood gases during the imaging experiments since frequent bleeding could result in hypovolemia. Instead, to evaluate for potential effects of chronic cocaine on blood gases we assessed the effects in a separate group of control mice (saline, ~0.1cc/100g/day, i.p. n=3) and chronic cocaine exposed mice (cocaine, 30mg/kg/day, i.p. n=3) pre-treated for ~ 4 weeks (for detail procedures, see Supplementary 6). In these mice, pH, pCO2 and pO2 did not differ between groups and remained normocapnic for both control and cocaine-exposed mice (Table S3).

Studies in the rat model

The experiments were performed in Wistar male rats, and the self-administration protocols and timelines for imaging are illustrated in Fig.2j. The animals were implanted with chronic intravenous (i.v.) catheters and chronically exposed to either saline (control group) or cocaine (ShA, LgA groups) for 26 consecutive days via intravenous self-administration. After first 7–9 days of cocaine self-administration, ShA rats received a daily 1hr session under a fixed-ratio schedule (FR1 with a 20s timeout) for the duration of ~17 days pretreatment period, whereas the LgA group received a daily 6hr session also under FR1, during this period. Groups were assigned to balance responding during first 7–9 days. For

this purpose, the rats were trained to self-administer i.v. saline or cocaine at a volume of 0.1cc/injection (saline) or at a dose of 0.5mg/kg/injection (cocaine). The details for surgery, training and self-administration procedures were as described previously (Wee et al., 2009).

Animals were imaged 7–8 days after their last self-administration session. During imaging, animals were anesthetized and ventilated with 1.5%–2% isoflurane mixed in a 60–70% O2/Air mixture. During the surgery, a femoral artery was catheterized for continuous arterial blood pressure monitoring and a femoral vein was catheterized for drug administration. The animal was then positioned in a stereotaxic frame (KOPF 900) to minimize brain motion. A cranial window (~6×4 mm^2) was created above the PFC (Anterior:+1 to + 5mm; Lateral: +/− 3mm from the bregma, (*33*)). The dura was carefully removed, and the exposed brain surface was immediately covered with 1.25% agarose gel and affixed with a 100μm-thick glass coverslip to maintain normal cranial pressure. After the surgery, the animal and the stereotaxic frame were transferred to the image suite for experiments. The animal's physiology was continuously monitored during the surgery and imaging, including electrocardiography, mean arterial blood pressure (MABP), respiration rate, and body temperature (Module 224002, Small Animal Instruments). For the first experiment only, rats underwent self-administration in Dr Koob's lab before transportation to Dr. Du's lab for imaging.

A home-built multimodal imaging platform was used to assess the effect of cocaine on CBFv networks and on oxygenated hemoglobin. 3D ODT was used to visualize angiography and quantify CBF in cortical neurovascular networks (Ren et al., 2012). It was integrated with a dual-wavelength imaging system (DWI) into a modified zoom fluorescence microscope (AZ100, Nikon) to allow for simultaneous imaging of CBFv and the oxygenated- and deoxygenated-hemoglobin concentrations within the surrounding tissue. For 3D ODT, a fast spectral-domain OCT system illuminated with a broadband source (λ_0=1.3μm, $\Delta\lambda$ 90nm; Inphenix) was coupled to the zoom microscope via a custom dichroic mirror (DM1) to provide an axial resolution of 8μm (Yuan et al., 2011). The collimated light beam (ϕ5 mm) exiting the sample arm of an optical coherence tomograph (OCT) was transversely scanned by a pair of servo mirrors (VM500, Cambridge Tech), focused with an achromatic lens (f40mm/0.1NA), which allows for a lateral resolution of ~ϕ12μm. The backscattered light from the brain was recombined with the reference light and detected by a high-speed linear spectrograph (a 1024-pixel InGaAs array; Goodrich, 47kHz). For the DWI system, two light emitting diodes (150 mW/each) at wavelengths of λ_1=570 nm and λ_2=630 nm were coupled into a ϕ3mm fiber bundle (NA/0.25) for illuminating the exposed cortex. The back-reflected light from the cortex encoded with the dynamic changes of total blood volume (Δ[HbT]) and deoxygenated hemoglobin (Δ[HbR]) was collected via the microscope optics (2×/0.22NA) and imaged by a 16-bit sCMOS camera (Zyla 5.5, Andor). Timesharing illumination scheme at λ_1 (=570 nm) and λ_2 (=630 nm) allowed for imaging at a frame rate of up to 16Hz/channel and both measures were then used to compute [ΔHbO$_2$] (Yuan et al., 2011), i.e.,

$$\begin{bmatrix} \Delta[HbO_2(t)] \\ \Delta[HbR(t)] \end{bmatrix} = \begin{bmatrix} \mathcal{E}^{\lambda_1}_{HbO_2} & \mathcal{E}^{\lambda_1}_{HbR} \\ \mathcal{E}^{\lambda_2}_{HbO_2} & \mathcal{E}^{\lambda_2}_{HbR} \end{bmatrix}^{-1} \cdot \begin{bmatrix} \dfrac{\ln\left(R_{\lambda_1}(0)/R_{\lambda_1}(t)\right)}{L_{\lambda_1}(t)} \\ \dfrac{\ln\left(R_{\lambda_2}(0)/R_{\lambda_2}(t)\right)}{L_{\lambda_2}(t)} \end{bmatrix} \quad (1)$$

where ε refers to the molar spectral absorptivity of a chromophore. $R_{\lambda_1}(t)$, $R_{\lambda_2}(t)$ are the diffuse reflectance images measured at these two wavelengths, and $R_{\lambda_1}(0)$, $R_{\lambda_2}(0)$ are their baseline values prior to the cocaine challenge. $L_{\lambda_1}(t) \approx L_{\lambda_2}(t)$ where $L_{\lambda_1}(t)$, $L_{\lambda_2}(t)$ are their pathlengths. The dynamic changes in [HbO$_2$] and in [HbT] were captured in response to a drug (e.g., cocaine challenge of 1mg/kg, i.v.) from both control and cocaine-exposed animals. ROIs in cortical areas where there were no visually distinguishable vessels were selected to compute [HbO$_2$] in cortical tissue at each time point for a total of 40min, including a 10min baseline measure before cocaine challenge and a 30min cocaine measure following its injection. For the comparisons between control and LgA animals, we integrated the changes in [HbO$_2$] over t=30min after the cocaine challenge and compared the means of [HbO$_2$] in the PFC between the groups. To analyze whether the 'low' [HbO$_2$] area in the PFC was correlated to the dose of cocaine consumed during self-administration, the mean area of [HbO$_2$] −10% was calculated from the [HbO$_2$] image for each LgA animal, which was used to compute the correlation with the total escalation doses of cocaine consumed by each animal.

To assess if cocaine-induced CBF decreases in the PFC in LgA rats was causally related to their compulsive-like cocaine intake, we evaluated whether the vasodilating effects of the L-type Ca^{2+} antagonist drug nifedipine in the PFC were associated with reduced cocaine intake and with changes in neuronal activity. Self-administration procedures were the same as above, including one saline group (n=6), one LgA group (n=5) and a second LgA group that received intraperitoneal (i.p.) nifedipine (20mg/kg, n=5; Araki et al. 1999) 20min prior to the initiation of the self-administration sessions. Groups were assigned based on responding during acquisition. After 14 days of self-administration rats were prepared for imaging 24hrs after the last session as described above. HbT was measured as above, while CBFv was calculated by reconstructing laser speckle flow image series by computing the speckle variances in both the spatial and temporal domains (Yuan et al., 2011, Chen et al., 2016). To assess the changes in neuronal activity, the [Ca^{2+}] indicator GCaMP6f was used. Three weeks prior to the commencement of experiments the rats were injected with AAV1.Syn.GCaMP6f.WPRE.SV40 virus (Penn Vector Core) into the right PFC (A/P: +3; M/L: 0.8) to induce GCaMP expression using procedures previously described (Gu et al, 2018). Two infusions of 0.5 μl were made (D/V: −1.4 and −1mm from skull) at a rate of 0.2μl/minute, and the injector was left in place for 20min following each infusion to allow for diffusion. Additionally to test whether nifedipine could prevent reinstatement of cocaine seeking in rats, a second experiment was performed. Following LgA self-administration, as described above, rats underwent extinction training (lever presses resulted in saline infusion, n=8) until they reached a criterion of less than 10 infusions in a session. Animals were tested for cocaine induced reinstatement 24 hrs after they reached extinction criteria. For reinstatement, animals were injected with 10 mg/kg (i.p.) cocaine immediately before they

were placed in the self-administration chambers. Prior to reinstatement animals were randomly selected to be pretreated 20 min prior to cocaine either with saline or with 20 mg/kg nifedipine, which was followed by an additional extinction session. The saline or nifedipine reinstatement sessions were counterbalanced and were 48 hrs apart separated by an extinction session.

For the non-contingent cocaine administration studies in rats, we used two groups of animals: one received daily i.p. injections of saline (0.7cc/100g/day) and another group received the cocaine (30/mg/kg/day) for 4 consecutive weeks (Supplemental Fig.S3O). Afterwards, in vivo imaging was conducted for each animal to record the CBFv change in the somatosensory cortex in response to an acute intravenous saline (0.1cc/100g, i.v., for control animals) or cocaine injection (1mg/kg, i.v., for cocaine animals). The somatosensory cortex was used as a control region to determine if the vasoactive effects of cocaine were specific to PFC or occurred in other cortical regions.

For the assessment of microvascular density and vascular endothelial growth factor (VEGF) in PFC and somatosensory cortex, we used ex vivo fluorescence measurement to quantify microvascular density in the brain of rats with or without chronic cocaine pretreatment (Supplemental 3, Fig.S2). Animals were divided into 2 groups: in the control group, the animals were treated with saline (0.7cc/100g/day, i.p., n=4) for 28 days (4 weeks), whereas the animals in the cocaine group were treated with cocaine (30mg/kg/day, i.p., n=6) for 4 weeks. FITC-Dextran (mol wt 2×10^6, 50mg/ml), a fluorescence dye, was intracardiacally infused (500μl) 1min before the animal was sacrificed to label the microvessels, and the whole brain of the rat was removed from the skull, then incubated in cold 4% formaldehyde solution (Thermo Fisher Scientific, Waltham, MA) overnight. The brain was then immersed in sucrose (99.5%, Sigma-Aldrich St Louis, MO) solution with increasing concentrations from 10% to 20% to 30%. The brain was cryosectioned, and the brain regions of the PFC and somatosensory cortex were imaged by fluorescence microscope (E80i, Nikon Instruments).

To visualize the VEGF expression, brain slices were bathed with 5% goat serum (ab 7481, Abcam) for 30min. The samples were then incubated with anti-VEGF antibody (ab 52917, Abcam) at 1:200 dilution for 1hr, followed by a further 1hr incubation with an Alexa Fluor® 488-conjugated Goat Anti-Rabbit IgG H&L (1:1000, ab150077, Abcam). The slices were finally mounted with Dapi Fluoromount-G® (SouthernBiotech, Birmingham, Alabama, USA) for imaging. ImageJ software was used to count microvascular density and to assess VEGF fluorescence density.

Studies in the Mouse Model

For the longitudinal imaging studies of the vascular tree and CBF we used C57BL mice. For studies of the PFC (Naïve n=5; cocaine, n=7, Fig. 5j), a cranial window was implanted on the frontal cortex (Lateral: 0.1 – 0.31mm; Anterior: 1.5 – 4.5mm from the bregma,(Paxinos et al 2004), and for studies of the somatosensory cortex (Naïve n=6; cocaine n=6, Fig. 6i), the cranial window was implanted on the sensorimotor cortex (Lateral: 0.25 – 2.75mm; Anterior: −0.25 – 2.75mm from the bregma,(Paxinos et al, 2004). Each mouse was anesthetized with a mixture of ~2.5% isoflurane in a 60–70% O2/Air mixture by a face

mask, and its head was then mounted on a customized stereotaxic frame, shaved, and cleaned with alcohol and iodine. For cranial window implantation, all surgical tools and supplements, operation area and cranial window cover glasses were sterilized by autoclaving and 70% alcohol. The cortical skin was removed and the fascia and connective tissue on the skull were cleaned with a hydrogen dioxide solution with q-tips. Once dried, the skull was thinned with a dental drill (Ideal Micro Drill; Roboz) and the bone was carefully removed over the region of interest (ROI). A drop of dexamethasone sodium phosphate (2mg/ml) was applied to the brain to prevent cerebral edema and the exposed brain surface was covered using a glass coverslip ($4\times3mm^2$, 100μm thick). The edge of the coverslip was sealed with glue and dental cement, which was spread around the coverslip to further secure its attachment to the skull. For post-surgery treatment, an anti-inflammatory drug was injected (Flunxin 2.5mg/kg, subcutaneous injection) every 12hrs for 2 days. The cranial window was implanted over the area of the PFC or the somatosensory cortex and was used to image the animals repeatedly during cocaine exposure and after cocaine discontinuation. After 3 days to allow for recovery from the surgery, saline was administered (~0.1cc/100g/day, i.p.) in the control animals and cocaine was administered (30 mg/kg/day, i.p.) in the chronic cocaine animals for ~28 days (Fig. 5j). The animals used for sensorimotor cortex were continuously imaged for an additional one month during withdrawal until day 60 (Fig. 6i).

We used a newly developed μOCA/μODT for 3D imaging of CBF networks in the mouse cortex. Similar to OCA/ODT, it can simultaneously image the cerebral microvasculature (μOCA) and quantify CBFv without the need for extrinsic labeling. In addition, it has dramatically enhanced blood flow detection sensitivity to facilitate fast, quantitative 3D capillary CBF imaging (e.g., resolving $\varphi<5$μm capillaries with a minute flow rate of <20μm/s) over a larger field of view (FOV; e.g., $3\times3\times1.5mm^3$). Our custom μOCA/μODT system is powered by an ultrabroad band light source ($\lambda=1310$nm, $\lambda_{FWHM}=220$nm) to allow for a high axial resolution (2.5μm), which is defined by the transform-limited coherence length, $L_c=2(ln2)/\pi\cdot\lambda_0^2/ \lambda_{cs}$. In the sample arm the collimated light was transversely scanned by a fast servo mirror and focused onto the mouse cortex through a cranial window with a $f16mm/NA0.25$ NIR objective yielding a lateral resolution of 3.2μm. The backscattered interference fringes spectrally encoding the depth profile were detected by a high-speed linescan InGaAs camera (2048-pixels, 145k-lines/s; GL2048, Sensors Unlimited) synchronized with sequential transverse scans for 2D/3D μOCT acquisition. The Doppler flow (phase subtraction) image and maximum intensity projection image were instantaneously processed by a graphic processing unit (GPU), permitting reconstruction of μODT images as fast as 473 fps for a B-scan containing 1k×2k pixels.(You et al., 2014, You et al., 2015).

Quantification of CBFv and angiogenesis: The blood flow velocity of cerebral vessels (CBFv) was determined from the ODT image based on Doppler flow reconstruction algorithms including the phase subtraction method (PSM) and phase intensity method (PIM) to enhance the blood flow detection (Zhao et al., 2000, Ren et al., 2012). To assess the vascular density, 3D OCA images were used. A binary operation was then applied to the OCA images to skeletonize the cerebral vascular networks using MATLAB software (Palagyi et al., 1998). Additionally, an adaptive rolling window method (window size:

50pixels × 50pixels) was utilized to provide a spatially resolved measure of vessel density from the skeleton of cortical vessels within the FOV. The local vascular density fraction was quantified using vascular fill factor (*FF*), which is defined as the ratio of the pixels occupied by vessels to the total pixel numbers within the rolling window, i.e.,

$$FF = \frac{Total\ vessel\ poixel\ \#}{Total\ pixel\ \#\ of\ ROI} \tag{2}$$

The spatial resolved density map can be obtained by rolling the adaptive window in both the vertical and horizontal directions with an increment step, e.g., a fraction of the window size *w* such as 0.1*w* in order to attain sufficient lateral resolution. Using this approach, the localization of the angiogenesis within the OCA image can be determined. In addition, the mean *FF* of the vessels over the FOV of the images can be calculated,

$$\overline{FF} = \frac{1}{M}\sum_{1}^{M} FF \tag{3}$$

where M represents the total number of ROIs selected in each image.

To assess vasoconstriction as a function of time during chronic cocaine exposure, the diameters of arteries and veins in the PFC (n=7) and in the somatosensory cortex (n=6) were tracked from μOCA images. For comparison with controls (n=5 for PFC and n=6 for somatosensory cortex) similar vessels were measured. The ROIs were selected randomly for each vessel type and the diameters of these selected vessels, i.e., $\phi(t_d)$ were traced every 7 days until the end of treatment with saline or cocaine (e.g., t_d=0, 7, 14, 21, 28 for PFC studies, and t_d=0, 3, 6, 9, 12, 15, 18, 21, 24 and 27, and additionally at 34, 41, 49 and 62 during cocaine withdrawal for the somatosensory cortex studies). The diameter change of the specific vessel was compared with its baseline, i.e., $\phi(t_o)$ before the saline/cocaine pretreatment, thus presenting as,

$$\Delta\phi(\%) = \{[\phi(td) - \phi(to)]/\phi(to)\} \times 100 \tag{4}$$

To analyze the CBF in constricted vessels and in the surrounding new vessels (angiogenesis), we measured their CBFv from the repeated μODT images obtained in the cocaine or saline exposed mice (controls) as a function of time, which were conducted for 28 days for PFC and for ~60 days for sensorimotor cortex studies, respectively.

Statistical Analysis

Statistical tests were performed with SYSTAT Software (Chicago, IL, USA). The differences in cerebral blood flow velocity (CBFv) and the vascular density between the control, ShA and LgA animals were tested by one-way ANOVA followed by post hoc tests (Student-Newman-Keuls) for inter-group comparison. Two-way repeated measures ANOVA was used to test the hypothesis of CBFv increases in newly grown vessels and the hypothesis of density increases, and the inter-group comparisons were performed using the Student-Newman-Keuls method. Pearson Product moment correlation analyses were used to assess the correlation between CBFv reduction and the cocaine consumed by the animals. The

correlation with cocaine intake was done both for the untransformed and also for the log transformed data, which was applied to show the increase in cocaine intake between control, ShA and LgA groups. In the mouse model, time courses for the CBFv changes were quantified and the comparisons with respect to the vessel's baseline measure (pretreatment) were assessed for within and between groups (controls vs cocaine groups) using a factorial repeated measures ANOVA. Significance was set at p<0.05. All data are presented as mean ± s.e.m.

Results:

We used a multimodal imaging platform to image vascular morphology (Fig. 1), CBF and oxygenated hemoglobin in the PFC and somatosensory cortex (used as comparison). μOCA was used to assess vascular morphology *in vivo* (Fig. 1b) and simultaneously μODT was used for quantitative three-dimensional (3D) imaging of CBFv networks (Fig. 1a, Fig. 1c), e.g., in arteries (red dashed-lines), veins (blue dashed lines) and capillaries (as illustrated in Fig. 1b). We used dual-wavelength optical imaging (DWI) to capture dynamic changes in oxygenated ([HbO2]) and deoxygenated hemoglobin ([HbR]) concentrations in response to cocaine administration (see Eq. 1). This was effected by switching between two wavelengths, λ_1=570nm (brown, equally absorbed in oxygenated and deoxygenated hemoglobin; Fig. 1d-e) and λ_2=630nm (red, absorbed more strongly in deoxygenated hemoglobin; Fig. 1f).

Studies in the rat model

Self-Administration Model: Both ShA (n=7) and LgA (n=6) rats were initially habituated to one hour daily of cocaine exposure for self-administration and compared to control animals (n=6; Fig. 2j). Controls animals were handled identically to the cocaine exposed rats (see Methods). After training, ShA rats were allowed to self-administer cocaine for 1hr/day (0.5mg/kg/ i.v., 7–8 mg/kg average total daily dose) and LgA rats for 6hr/day (50–60 mg/kg average total daily dose; Fig. 2d). LgA rats have been shown to escalate cocaine intake with time, to develop hedonic tolerance and withdrawal, and show compulsive-like responding for cocaine (Koob 2009). ShA rats do not show any of these changes but keep a stable day to day self-administration pattern with no escalation (Koob 2009). Consistent with the literature, LgA rats, in the present study, escalated their cocaine self-administration (Fig. 2d).

Fig. 2 shows images of quantitative CBFv and of angiography in the PFC of a LgA animal (Fig. 2e,f) and of a yoked control (i.e., saline infusion instead of cocaine; Fig. 2a,b). The CBFv in the PFC of the LgA animal (Fig. 2e) was much lower than that of the control (Fig. 2a) throughout the cerebrovascular tree. Three group comparisons (one way ANOVA) of CBFv in middle-size vessels (i.e., diameter φ=124.27±16.09μm) showed a significant difference between groups [F(2,35) = 3.33, p = 0.047]. Post hoc t-tests revealed that controls (8.99±1.02mm/s) had significantly faster CBFv than LgA rats (6.39±0.48mm/s; p=0.043; Fig. 2h) whereas CBFv in ShA rats (n=7) did not differ from controls (p=0.180; ShA: 7.18±0.56mm/s, p = 0.078) nor from LgA rats (p = 0.43) suggesting an intermediate state between the two groups (Supplemental 2, Fig.S1). Correlation analyses between CBFv in

the PFC and the doses of cocaine self-administered by the animals (in $\log_{10}(\text{mg/kg})$ scale) revealed a significant inverse association (Fig. 2k), such that rats with the greater reductions in CBFv in the PFC consumed the largest doses of cocaine (r=−0.6, p=0.008; The correlation with the untransformed values (raw cocaine intake) with CBFv changes showed a similar association (r=−0.45, p=0.054). The correlation with the raw data was significant also when we controlled for the non-normal distribution using Spearman rank order correlation (rs = - 0.48, p = 0.037).

To evaluate whether CBFv reductions triggered changes in vascular density, we quantified the vasculature present over the FOV in the µOCA images. We computed vascular density as the mean vascular fill factor (i.e., as described in Eq.3), which was defined as the ratio of the number of pixels occupied by vessels to the total number of pixels in the FOV (see Eq.2 for detail). To do this, the OCA images were skeletonized where each vessel and its branches were simplified into lines (Palagyi and Kuba, 1998). Fig. 2c and Fig. 2g exemplify the skeletonized vasculature maps of a control (2c) and a LgA (2g) animal. values (one-way ANOVA) differed significantly between groups [F(2, 35) = 5.56, p = 0.008]. LgA rats (0.035±0.0015, n=6, ROIs=12) had a significantly greater density than controls (0.029±0.0008; n=6, ROIs=12; p =0.011, Fig. 2i) and ShA rats ($\overline{FF}$=0.030±0.0015; n=7, ROIs=14; p =0.011). There was no difference between ShA rats and controls (p = 0.96; Fig.S1d).

To assess whether cocaine-induced disruptions of vascular networks in LgA animals were exacerbated during cocaine intoxication, we measured the hemodynamic response in the PFC to an acute cocaine challenge (1mg/kg, i.v.). Spectral images at λ_1, and λ_2 were acquired using a time-sharing approach (i.e., 12.5Hz sampling rate for each channel) continuously during the baseline period and up to 30min after cocaine administration. Changes in [HbO_2] and [HbR] in cortical tissue were calculated from the time-lapse images at λ_1, and λ_2. (Eq. 1; Yuan et al., 2011). Fig. 3a,b illustrate the spatiotemporal changes of [HbO_2] in the PFC in response to acute cocaine in a control and an LgA animal, respectively. These images show that in the control, [HbO_2] decreased within 3 min after cocaine injection, predominantly around the central vein above the middle PFC, and this decrease was short lasting and recovered within a few minutes of cocaine injection (Fig. 3a). In contrast, the [HbO_2] decrease in the LgA animal (Fig. 3b) was longer lasting (persisted >20min post-cocaine injection) and widespread (observed over the full FOV). Group comparisons of the time courses for cocaine-induced decreases in [HbO_2] showed that for controls (green curve, n=6), acute cocaine abruptly decreased [HbO_2] within t=2min of injection with peak decreases occurring at ~3min post injection (−10.7%±1.2; p=0.001), and with 60–70% recovery by t=6–9min and complete recovery by t≈18 min; whereas for LgA animals (red curve, n=6), peak decreases occurred at ~4min post cocaine injection, were larger (−20.7%±3.8; p=0.002) and did not recover until t=29min post cocaine (Fig. 3c). The peak decrease in [HbO_2] triggered by cocaine was significantly stronger (p=0.024) and the duration longer for LgA than for controls (p=0.02).

We also compared the magnitude of [HbO_2] in response to acute cocaine by integrating [HbO_2] over t=30min after cocaine challenge. Decreases in [HbO_2] in LgA animals were ~1.4 times greater than in controls (p=0.02, Fig. 3d). To assess the correlation between the

doses of cocaine self-administered by LgA animals and the magnitude of the Δ[HbO$_2$] decreases triggered by acute cocaine, we computed the percentage of the area with 'low' HbO$_2$ (i.e., percentage of pixels with Δ[HbO$_2$] $\leq$ −10.0% with respect to the total number of pixels in the FOV) and showed that it was significantly correlated with the cocaine doses administered (r=0.98; p<0.001; Fig. 3e).

Non-Contingent Administration Model: To assess if cocaine's effects in CBFv and HbO$_2$ were specific to the self-administration model and/or the PFC we evaluated the effects of an acute cocaine injection (1 mg/kg, i.v.) in the somatosensory cortex in rats exposed chronically (4wks) to non-contingent cocaine administration (30/mg/kg/day, i.p.). Responses were compared those after an acute i.v. saline injection in animals exposed chronically (4wks) to saline (0.7cc/100g/day, i.p, Fig.S2o, Supplemental 3). The effects of non-contingent cocaine administration in the somatosensory cortex were similar to those in the PFC obtained with the self-administration model. Fig.S2B0-B3 illustrated the CBFv changes in response to acute cocaine (1mg/kg, i.v.) in the chronically cocaine exposed rats, Fig. S2B4 showed a decrease within t=2–6min post cocaine injection to ΔCBFv=−18.8± 6.2% from baseline (i.e., t 0min, p=0.039,), which was long lasting and did not fully recover by 28min post injection. Acute saline injection in the control animals did not change CBFv (Fig.S2A0-A4).

Ex-vivo histochemistry analyses showed that the microvascular density, using fluorescence labeling (FITC-Dextran fluorescence indicator), was significantly increased in the cocaine treated rats (4 weeks, non-contingent i.p cocaine 30mg/kg/day) both in somatosensory cortex (p=0.007; controls: 287.3±24.5/mm^2; chronic cocaine: 379.4±23.2/mm^2; Fig.S2c-e), and in PFC (p=0.009; controls: 246.4±17.7/mm^2; chronic cocaine: 298.4±7.5/mm^2; Fig.S2i-k). We also showed that the vascular endothelial growth factor (VEGF), a potent angiogenic factor involved in blood vessel growth, was increased both in the somatosensory cortex (p=0.006; controls: 0.45±0.13; chronic cocaine: 1.32±0.3; Fig.S2f-h) and in the PFC (p=0.006; controls: 0.38±0.07; chronic cocaine: 1.06±0.2; Fig.S2l-n). These results indicate that angiogenesis occurred in the brains of animals exposed to chronic cocaine as shown by the increase in vessel density and VEGF levels when compared to controls.

L-Type Ca^{2+} blockade improved cocaine-induced [Ca^{2+}] and CBF changes in PFC and reduced cocaine intake

In a separate self-administration experiment, we injected rats with a virus expressing GCaMP6f (a calcium indicator) to measure neuronal activity concurrent to CBFv changes (Fig. 4). To assess if preventing the hemodynamic disruption triggered by cocaine would prevent escalation of cocaine intake we evaluated the effect of nifedipine (L-type Ca^{2+} antagonist and vasodilator) pretreatment in LgA animals. We hypothesized that by dilating vessels and ensuring adequate blood supply, PFC dysfunction would be diminished, which in turn would protect against escalation of cocaine intake. Fig. 4E illustrates the study protocol. Briefly, nifedipine (20mg/kg, i.p.) was given 30min prior to each self-administration session to LgA rats (LgA-NIF) and cocaine intake, CBF, and [Ca^{2+}] reactivity in PFC, were compared to that in LgA animals not given nifedipine (LgA). Nifedipine pretreatment significantly reduced cocaine intake over 14 days of self-

administration ([F(1, 8)=10.81, p=0.011]; total intake over self-administration LgA=1074.4±46.65, n=5; LgA-NIF=779±76.8, n=5; Fig. 4F). And the reduction in cocaine intake was observed starting form the first pretreatment session and was consistently reduced throughout all of the sessions (Fig 4G). When imaged after self-administration, LgA animals showed significantly greater changes in neuronal intracellular calcium ($[Ca^{2+}]$) in response to cocaine than controls or LgA-NIF rats [F(2,13)=4.53, p=0.032], Fig. 4D). This group difference was due to an increase in peak calcium in LgA rats compared to controls and LgA-NIF rats ([F(2,13)=7.49, p=0.007]; control=4.3±0.22, n=5; LgA=6.35±0.71, n=5; LgA-NIF=3.24±0.64, n=5; Fig. 4H). Finally, nifedipine also prevented the reductions in CBF following chronic cocaine administration (Fig. 4I). Specifically whereas LgA rats showed a significant reduction in CBFv in the PFC following self-administration, LgA-NIF rats did not ([F(2,13) =4.83, p=0.027]; control = 22883.9±749.3, n=6; LgA=16831.89±1831.4, n=5; LgA-NIF= 24734.56 ±2726.6, n=5). It is important to note that animals were imaged 24 hours after the last dose of nifedipine, which has a 2 hour elimination half-life, and therefore the differences compared to the non-nifedipine group are due to a blockade of cocaine-induced adaptations rather than a lingering effect of the medication. Furthermore, as both changes in CBF and neuronal activity were prevented by nifedipine, one could speculate that reductions in CBF contributed to neuronal dysfunction.

Disruption of the PFC is known to contribute to relapse in humans and thus we hypothesized that nifedipine by counteracting the vascular and neuronal effects of cocaine in the PFC would prevent reinstatement in the rats. To test this hypothesis, we assessed the effects of nifedipine in the cocaine priming model of reinstatement (McFarland and Kalivas, 2001). After extinction rats were treated either with saline or with 20 mg/kg of nifedipine prior to priming them with cocaine (10mg/kg ip) for reinstatement. A repeated measures ANOVA found a significant effect of session type on number of infusions [F(3,19) = 33.76, p<0.001] (Fig. 4J). Post-hoc analysis showed a significant decrease in the number of infusions from the end of self-administration (78.25±5.08) to the end of extinction (7.13±1.76, p<0.001). When pretreated with saline, priming with cocaine significantly increased infusions (RI+V: 41.29±10.63, p=0.001, Fig. 4J) relative to the end of extinction, consistent with cocaine-induced reinstatement. In contrast when pretreated with nifedipine, priming with cocaine did not significantly increase infusions (RI+N: 18.71±5.19, p=0.19, Fig. 4J) relative to extinction. Infusions after cocaine priming were significantly higher when rats were pretreated with nifedipine than when they were not (p=0.01). These results suggest that even when administered after chronic cocaine use, nifedipine may have beneficial effects at preventing cocaine-induced reinstatement of drug seeking.

Studies in the Mouse Model

The mouse model allowed us to perform longitudinal imaging for 30–60 days (Park et al., 2015) and hence measure the changes in vascular density and CBFv as a function of chronicity and detoxification. For this purpose, we implanted a cranial window over the PFC (or the somatosensory cortex; see below) of the mouse's brain and concurrently acquired μOCA for 3D angiography and μODT for quantitative 3D CBFv networks at high spatial resolution (~3μm) over a relatively large FOV ($2.5×2.5×1.2mm^3$).

The control group (n=5) received a daily injection of saline (~0.1cc/10g/day i.p.); the cocaine group (n=7), received a daily injection of cocaine (~30mg/kg/day i.p.) for 28 consecutive days. Animals were periodically scanned with μOCA/μODT (Fig. 5j). Fig. 5a-c show the time-lapse 3D μOCA images of vascular networks in PFC from a control animal on Days 0, 14, and 28, and Fig. 5d-f show the images from a cocaine animal. For illustration purposes, 'large' (φ~150μm, "1") and 'middle' (φ~100μm, "2") sized vessels were selected in the control and the cocaine mice (Fig. 5a,d) for comparison. In the control animal neither vessel '1' nor '2' changed between day 0 to day 28 (Fig. 5a versus Fig. 5c), whereas in the cocaine animal the vessels' diameters decreased from φ~150μm to φ~80μm for vessel '1', and from φ~100μm to φ~40μm for vessel '2' (Fig. 5d,f). Fig. 5k shows the time courses for the diameter changes of arteries and veins in controls (n=5, total ROIs=40) and in cocaine animals (n=7, total ROIs=56) during saline or cocaine exposure over day 0 to day 28. The diameters of both arteries and veins decreased significantly as a function of cocaine chronicity (Fig. 5k).

In cocaine treated animals, the mean diameters of arteries and veins decreased from baseline (prior to cocaine) to the end of treatment (28 days). For the arteries, the diameter was reduced 21.1±9% (from 57.8±37.8 μm to 42.6±25.0μm) and two-way repeated measure ANOVA test showed a significant difference between cocaine-treated animals and controls as a function of time (i.e., $[F_{(4,40)}=7.467, p=0.0001]$; Fig. 5k). For the veins, the diameter was reduced 30.3±17% (from 71.5±44.2μm to 48.0±29.3μm; $[F_{(4,40)}=5.822, p=0.0008]$, Fig. 5k), and changes in vessel diameter differed significantly from those in controls ($p<0.05$). Figure 5l summarizes the comparison of the cocaine-treated animals to the controls (saline-treated animals) after 28 days of treatment. A separate one-way ANOVA shows that the diameter reductions induced by chronic cocaine exposures were significantly different from control, both for arteries (Coc_A vs $Saline_A$, $[F_{(1,40)}=9.982, p=0.01]$) and veins ($Coc_V$ vs $Saline_V$, $[(F_{(1,40)}=12.452, p=0.005]$).

To determine the relationship between cocaine-induced vasoconstriction and the formation of new vessels, we assessed the temporal sequence between these two events. Fig. 5g-i show the 'zoom-in' ROIs within Fig. 5d-f (marked as yellow boxes) to emphasize the progression of angiogenesis around the area of vasoconstriction (e.g., vessel '2', pink tracks). Angiogenesis appears as an extension of branches from preexisting vessels (e.g., vessels '3' and '4', green tracks shown in Fig. 5g) that surround the constricted vessel from day 0 to day 28 of cocaine infusions. It shows that, these two events were co-localized. To characterize the CBFv in the constricted vessels and in the surrounding area of angiogenesis, we selected 4–5 ROIs along the length (axis) of vessels '2' and '3' to track the CBFv changes as a function of time (for details, see Fig.S3). As illustrated in Fig. 5m, the diameter of vessel '2' decreased as a function of cocaine treatment days (dashed line, C2 in Fig. 5m), as did CBFv (pink line, C2 in Fig. 5m), from 9.28±1.12mm/s at baseline (day 0) and 9.95±2.27mm/s on day 7, to 6.35±1.34mm/s on day 14, 3.35±0.53mm/s on day 21, and 2.20±0.22mm/s on day 28. Note that in the 'growing' vessel branch '4', CBFv gradually increased from 4.50±0.96mm/s (green line, A1 in Fig. 5m) on day 0 to 7.43±2.23mm/s on day 7 and 7.98±2.18mm/s on day 14, and then plateaued at 8.70±0.93mm/s on day 21 and 8.77±1.39mm/s on day 28. These results show that, while cocaine-induced vasoconstriction decreased CBFv, it stimulated angiogenesis, which helped compensate for some of the

CBFv decreases. However, the slope of the CBFv decrease (k_c) in the constricted vessel (e.g., '2') from day 7 to day 28 is greater than that of the CBFv increase (k_a) in the angiogenetic vessel (e.g., '4'), i.e., $|k_c| \approx |{-}0.38| > |k_a| \approx 0.07$ (Fig.S3B, Supplemental 4). This suggests that, although angiogenesis might ameliorate to a certain extent cocaine-induced CBFv reductions in the PFC, this compensation is insufficient to return CBFv to baseline values.

To assess if cocaine-induced changes in the vasculature and its hemodynamics were specific to the PFC or occurred in other cortical regions, and to assess if it recovered during withdrawal, we evaluated cocaine's effects on the somatosensory cortex longitudinally over a 2-month period, during which time mice were non-contingently exposed to cocaine for 4wks (i.e., 28 days) and then withdrawn for the following 4–5wks (i.e., 30–32 days). A cranial window was implanted over the sensorimotor cortex in controls (saline, ~0.1cc/10g/day, i.p., n=6) and in chronic cocaine exposed animals (cocaine, 30 mg/kg/day, i.p., n=6). Using the same protocol as for the PFC (described above) we obtained μOCA (angiographic) and μODT (quantitative CBFv) images periodically over 60 days to track their changes with time (Fig. 6i). Repeated μOCA and μODT images obtained before cocaine (i.e., 0-day) and after 12 and 27 days of cocaine exposures followed by withdrawal for 22 days are shown in Fig. 6a-h. 'Pink' arrows highlight some of the constricted vessels, and green circles mark the surrounding angiogenic vessels growing with time. It shows that chronic cocaine also induced vasoconstriction (e.g., pink arrows in Fig. 6a-h) in the somatosensory cortex. Similar to what was observed in the PFC, angiogenesis (including angiogenetic capillary nets) developed in the areas surrounding constricted vessels (green circles Fig. 6c). Note that, although angiogenesis occurred on Day 12 (as shown in Fig. 6c), there was little detectable blood flow in these vessels (green arrows pointed in Fig. 6d). This is likely the result of these new vessels being nonfunctional, e.g., unable to properly recruit blood flow. After 12 days of cocaine-induced vasoconstriction the new micro-angiogenic vessels started to show perfusion and micro-vessel collateralization was observed (as illustrated in Fig. 6e-f). Quantitative analysis of the diameters of neovessels is provided in Supplemental material (Fig.S4I, Supplemental 5). The analysis indicates that angiogenesis in cortex developed in the capillary bed early on (e.g., on Day 12) and was followed by micro-vessel collateralization later on (e.g., after 27 days of cocaine exposure). These neovessels were located within brain tissue (Fig.S4II). Both the reduced vessel diameter and increased density were long lasting and persisted for at least 22 days after cocaine treatment discontinuation (i.e., from Day 27 to Day 49 as shown in Fig. 6e,g, pink arrows).

Fig. 6j shows cocaine-induced diameter changes in individual arteries and veins, as well as the hemodynamic (i.e., CBFv) changes in neovessels. It indicates that the response of arteries (red dashed lines) to cocaine varied from vessel-to-vessel, with some decreasing their diameter by 50–70% whereas others did not change. In contrast, cocaine consistently decreased the diameter of veins (blue dashed lines). The mean diameter changes in both arteries (red solid line) and veins decreased as a function of time. The angiogenic vessel started to recruit flow starting on Day 10–12 and CBFv increased as a function of time and plateaued after Day 25 (green curve).

The CBFv changes in arteries and veins across different animals (n=6) are summarized in Fig. 6k. Fig. 6k shows that after 21 days of cocaine treatment, CBFv decreased ~28.08% in arteries (from 13.07±0.92 mm/s to 9.40±1.95 mm/s) and ~38.42% in veins (from 10.32±0.56 mm/s to 6.36±0.94 mm/s) with respect to baseline (day 0). The local CBFv in these vessels did not change from the last day of cocaine exposure (i.e., day 27) to after 25 ±5.4 days of cocaine abstinence (arteries: 8.08±1.3mm/s versus 9.0±2.1mm/s, p=0.942; veins: 6.02±0.64m/s versus 7.73±1.53mm/s, p=0.127). This indicates that the hemodynamic dysfunction did not recover following 4 weeks of cocaine abstinence.

The development of angiogenesis, reflected by neovessel D/D (black traces) and their flow recruitment reflected by CBFv (red traces) as a function of time is shown in Fig 6l. It indicates that neovessels grew rapidly after day 7–8 and reached a peak about day 12, after which they plateaued (ROIs=15). CBFv in these neovessels appeared with a 1–2 day delay from the time they were formed and then increased up to 3.46±0.74mm/s around day 27. However, the CBFv in these neovessels did not change further after cocaine withdrawal (from day 28 to day 62), indicating that the neovessels' capability for flow recruitment was limited and unable to fully compensate for the local CBFv decrease. Comparison of vascular density (assessed by) between baseline (day 0) and after 21 days of cocaine in Fig. 6m showed that of medium vessels (i.e., MID) increased from 0.02±0.002 to 0.03±0.006 (p=0.026) and of small vessels including capillaries (CAP) increased from 0.12±0.021 to 0.20±0.019 (p=0.035).

Discussion:

Here, we document significant vasoconstriction in arterial and venous blood vessels along with CBF reductions in the PFC, both in rats that showed compulsive-like self-administration of cocaine and in mice non-contingently exposed to cocaine chronically. The vascular and hemodynamic changes occurred early in the history of cocaine administration (within 1 week of cocaine exposure) and persisted after cocaine discontinuation. The longitudinal assessment allowed us to map the dynamic nature of the changes triggered by cocaine self-administration and revealed that cocaine induced significant vasoconstriction, which triggered proliferation of local blood vessels. However, despite the angiogenesis, CBF remained significantly reduced even after ~1 month of detoxification. Finally, we found that the CBF reductions in the PFC cooccurred with increases in neuronal reactivity to cocaine (as assessed with neuronal intracellular calcium ($[Ca^{2+}]$)), and that pretreatment with the calcium channel blocker nifedipine prevented both these changes in the PFC and reduced cocaine intake and blocked cocaine-primed reinstatement.

Our findings of reduced CBF in the PFC of LgA rats, and passively treated mice, are consistent with clinical findings of reduced CBF in the cortex (most prominently in the PFC) of cocaine abusers (Volkow et al., 1988). Our results illuminate the clinical findings by demonstrating that cocaine-induced reductions in CBF are due to cocaine-induced vasoconstriction of both arterial and venous blood vessels. Studies on isolated cerebral arterioles have shown that application of cocaine or its metabolites induced vasoconstriction, documenting a direct effect of cocaine on blood vessels (He et al., 1994). Most studies of vasoconstriction of cerebral vessels report on diameter reductions of arterial vessels, but

previous studies have also shown that venous vessels undergo vasoconstriction (Shen et al., 2013). In the present study, the cocaine-induced diameter decreases were observed across different types and sizes of vessels, including arteries, veins, arterioles and venules. Our studies also reveal that the vasoconstricting effects of cocaine in cerebral vessels are sensitized with repeated administration. It is possible that the mechanisms underlying the changes in vessel diameters differ for arteries and veins, as well as a function of vessels' size. For arteries that are large enough to have sympathetic innervation, the decreases might reflect vasoconstriction, whereas the diameter decreases in veins are likely to reflect both adaptations to accommodate for reduced flow to the tissue, as well as direct vasoconstricting effects of cocaine (Sofuoglu et al., 2001; Abbound et al., 1964). Though mechanism(s) are unclear, there is evidence that norepinephrine (NE) and L-type Ca^{2+} channels may play a role in the sensitization of cocaine-induced vasoconstriction (Laporte et al., 1997; Kalsner, 1993).

Cocaine's effects in cerebral blood vessels might also be due in part to its dopaminergic effects as demonstrated by the fact that dopamine D2 receptor blockade with haloperidol blocked cocaine induced vasoconstriction (He et al., 1994). Dopamine transporters (pharmacological targets of cocaine) are expressed in cerebral blood vessels (Ohtsuki et al., 2010), and cocaine could increase dopamine from dopamine terminals in close contact with arterioles and capillaries, resulting in vasoconstriction (Krimer et al., 1998). Cocaine's noradrenergic enhancing actions are also likely to contribute, for NE also has vasoconstricting effects (Giessler et al., 2002). While vasoconstriction would reduce CBF, further reduction could be produced by the neuronal effects of cocaine (Ren et al., 2012) through neurovascular coupling. Indeed, we previously reported that acute cocaine (1mg/kg, i.v.), depressed the resting-state field potentials of neurons, which correlated with decreases in CBF (Chen et al., 2016). This suggests that cocaine-induced reduction in synchronized spontaneous neuronal activity might also contribute to CBF reductions. However, in the LgA animals we observed that acute cocaine accentuated the CBFv decreases despite it enhancing the neuronal intracellular calcium ($[Ca^{2+}]$) increases, consistent with the disruption of neurovascular coupling by chronic cocaine exposure (Chen et al., 2018).

Our findings also revealed sensitization to cocaine-induced CBF reductions, such that an acute cocaine challenge triggered significantly larger and longer-lasting CBF reductions and [HbO$_2$] decreases in LgA than in drug naïve or in ShA animals. Indeed, in LgA animals, cocaine-induced decreases in [HbO$_2$] were double the magnitude of those in controls (20.7% versus 10.8%). In humans, studies using infrared optical imaging revealed that decreases in brain oxygen saturation of 13% were associated with EEG changes characteristic of cerebral ischemia in 97% of cases (Al-Rawi et al., 2006). Considering that chronically exposed animals had a 20.7% reduction in [HbO$_2$] when exposed to an acute cocaine dose, this indicates that these reductions are well within values associated with ischemia. Occurrence of ischemia with chronic cocaine exposure is consistent with clinical reports of transient ischemic attacks (TIA) and cerebral strokes in cocaine abusers (Sordo et al., 2014; Toossi et al., 2010; Treadwell and Robinson, 2007) and with preclinical findings of TIA in mice exposed chronically to cocaine (You et al., 2017).

We also documented angiogenesis with chronic cocaine exposure in both the rat and the mouse, in both self-administration and non-contingent administration models, indicating that this is not a unique phenomenon limited to a specific animal model or species. Furthermore, using the longitudinal mouse model we show that cocaine-induced vasoconstriction and the associated reductions in CBF in the PFC were regionally and temporally associated with the formation of new vessels. Similar findings were obtained in the somatosensory cortex, indicating that cocaine-induced vascular and hemodynamic changes are not specific to the PFC and occur in other cortical regions. Cortical CBFv remained significantly reduced even after 2530 days of cocaine withdrawal and despite the occurrence of angiogenesis.

Ischemia is known to trigger angiogenesis (Hayashi et al., 2003), so it is likely that cocaine-induced ischemia underlies the angiogenesis we observed with chronic cocaine in both the rat and the mouse models and in the PFC and somatosensory cortex. Moreover, the regional and temporal correspondence between cocaine-induced vasoconstriction and angiogenesis in the surrounding area is consistent with the hypothesis that cocaine-induced ischemia triggers angiogenesis.

Though the mechanisms by which hypoxia triggers angiogenesis with chronic cocaine exposure are unclear, we showed that in rats chronic cocaine increased the levels of vascular endothelial growth factor (VEGF) in somatosensory cortex after 2 and 4 weeks of cocaine exposures (Zhang et al., 2016). In the current study, we also show that microvascular density and VEGF are increased in the PFC after 4 weeks of cocaine exposure (Fig.S2(I-K) and Fig.S2(L-N)). Consistent with these results, clinical studies have reported increases in VEGF in the pleura of cocaine abusers (Strong et al., 2003). Since VEGF is a potent angiogenic factor involved in blood vessel growth in ischemic diseases (Marti et al., 2000), increases in VEGF could underlie the angiogenesis we observed in the PFC and somatosensory cortex of animals chronically exposed to cocaine. Though VEGF triggers endothelial cell proliferation and enhances vascular permeability in nascent vessels, newly formed vessels are immature and leaky (Zhang et al., 2002), which could explain why CBF remained decreased in the cocaine-exposed animals despite the increases in vessel numbers.

The longitudinal mouse model and our recently developed µOCA/µODT imaging tools enabled us to image vascular networks during 28 days of cocaine exposure and following one month of abstinence (Fig. 6i). The longitudinal analyses revealed that angiogenesis occurred around day 10 of cocaine exposure but then, along with CBFv, stabilized after ~25 days of cocaine administration. Noteworthy was the fact that CBFv remained unchanged following 30 days of cocaine abstinence and remained significantly reduced relative to the baseline, indicating that after the initial burst of angiogenesis there was little further recovery. Indeed, even after ~30 days of cocaine withdrawal and concomitant with an overall increase in vascular density, CBF remained significantly reduced. Such a time course implies that angiogenesis was insufficient to compensate for cocaine-induced disruption of cerebrovascular function and that cocaine-induced vascular and hemodynamic changes are long lasting and persist even after cocaine discontinuation. The lack of recovery in CBF despite the presence of angiogenesis could indicate that these new vessels are not functioning optimally. Clinically, it has been reported that in cerebral ischemic stroke there is active angiogenesis, which is most prominent in the penumbra (Krupinski et al., 1994;

Wei et al., 2001), and greater neovascularization was associated with longer survival (Krupinski et al., 1994). However, the number of microvessels filled with blood cells was significantly lower in the infarcted hemispheres, and the functionality of the new cerebral vessels following an ischemic event is unclear (Hayashi et al., 2003). Nevertheless, more attention is needed to investigate the role of angiogenesis, which could determine neuronal survival after cerebral ischemia, in the recovery of cocaine abusers.

In LgA rats we observed significant changes in vasoconstriction, CBFv and angiogenesis, and although a similar direction of effects was observed in the ShA rats, they were not significant. However, they were in the same direction and intermediate between values in LgA and controls. This indicates that there is a dose effect for cocaine-induced vascular toxicity. The fact that these same responses were observed in the mouse model, where animals were exposed to chronic cocaine non-contingently, indicates that these are direct pharmacological effects of cocaine and not dependent on the animal model used for exposure. These results also show that they occur both in rats and mice (and in humans as evidence by clinical studies); and are not specific to PFC since they occurred also in somatosensory cortex (presumably other cortical areas).

By measuring neuronal Ca^{2+} (marker of neuronal function) in the present study we were able to demonstrate that the PFC in LgA animals was hyper-responsive to an acute cocaine challenge. In our study this enhanced reactivity (reflected by an increase in neuronal Ca^{2+}) occurred concurrently with the reduction in CBF. In fact, when the rats were treated with nifedipine, a vasodilator and L-type Ca^{2+} channel antagonist, prior to self-administration sessions, both the reduction in CBF and neuronal hyper-responsiveness were blocked. Prevention of these adaptations was associated with reduced cocaine intake, suggesting that CBF hypofrontality and neuronal sensitivity contribute to compulsive-like drug taking in the LgA model. Indeed, it has been reported that escalation of drug intake is associated with loss of PFC neurons (George et al., 2008), which could potentially result from the ischemic effects of cocaine. Similarly, hypoactivity of the prelimbic cortex was associated with increased cocaine seeking, which was reduced by optogenetic stimulation of the PFC (Chen et al., 2013). Thus, one hypothesis is that by preventing the ischemic consequences of cocaine-induced vasoconstriction in the PFC its dysfunction can be minimized, and escalation of cocaine intake can be diminished. Similarly strengthening PFC function might provide resistance to relapse and account for the reduced cocaine-primed reinstatement we observed with nifedipine pretreatment. A similar disruption of cocaine-induced reinstatement (also cue induced reinstatement) was reported with fendiline pretreatment, which is another L-type channel blocker (Cunningham et al. 2015). Note, nifedipine could be altering cocaine intake by mechanisms other than vasodilation and recovery of PFC function. In cocaine users nifedipine attenuated the subjective effects of cocaine (Muntaner et al 1991) and in rodents it blocked the rewarding effects of cocaine as measured by conditioned place preference (Calcagnetti et al 1995). Therefore, it is possible that nifedipine reduced cocaine intake in part by interfering with its rewarding effects. Alternatively, Ca^{2+} antagonism may interfere with cocaine-induced synaptic plasticity and contextual memory (Degoulet et., 2016), which could also affect cocaine intake and reinstatement. Together these findings indicated that nifedipine may be beneficial both in preventing cortical damage and developing addiction when administered early, as well as

aiding in the maintenance of recovery in those with a history of abuse. Because the noradrenergic enhancing effects of cocaine contribute to its vasoactive effects it is possible that combining a drug that attenuates NE signaling (i.e., α1 antagonist α2 agonist) with an L-type calcium channel antagonist might enhance its beneficial hemodynamic and functional effects in the PFC and merits further investigation.

Our findings are clinically relevant, as they indicate that the sensitized reactivity of cerebral blood vessels to chronic cocaine will place cocaine abusers at increased risk of cerebral ischemia, particularly during cocaine intoxication. The increase in vascular density observed in our study may reflect an attempt to compensate for cocaine-induced hypoxia. Inasmuch as the PFC is a main target for cocaine-induced neurovascular toxicity, improving CBF may be particularly beneficial in helping recover executive function and self-regulation. Clinical and preclinical studies of chronic cocaine exposure have corroborated a decrease in PFC function, which has been associated in animals with compulsive-like cocaine intake and in humans with relapse and impairments in cognitive performance (Buttner, 2011). The mechanisms underlying hypofrontality are poorly understood, although there is evidence that adaptations in striato-cortical dopamine neurotransmission are involved (Volkow and Morales, 2015). However, the present study suggests a contribution of cocaine-induced cerebrovascular pathology to hypofrontality. Our finding that the [HbO$_2$] decreases in the PFC triggered by cocaine were associated with the amount of cocaine self-administered by animals suggests that hemodynamic deficits are involved in hypofrontality and thus likely to contribute to the escalation of cocaine intake in addiction.

One of the limitations of this study is that the measures of vasoconstriction were recorded while imaging the animals under isoflurane anesthesia. Isoflurane could have counteracted the vasoconstriction from cocaine since it produces cerebral vasodilation (Maekawa et al., 1986), so it is possible that cocaine-induced vasoconstriction might have been attenuated by the anesthesia. Another limitation is that optical imaging has limited penetration so we could not access subcortical brain regions. It would have also been desirable to obtain additional measures to characterize the impact of the hemodynamic changes triggered by cocaine such as disruption of blood brain barrier permeability, neuroinflammation or white matter changes.

In summary, here we document significant changes in perfusion in the PFC associated with chronic cocaine exposure that persist after cocaine discontinuation and are associated with altered neuronal activity. These findings show that cocaine-induced vasoconstriction, and the resulting ischemia, together with abnormal neuronal reactivity induced by cocaine contribute to PFC dysregulation that is associated with compulsive cocaine intake. We also show that nifedipine pretreatment attenuated the hemodynamic and neuronal changes in PFC from chronic cocaine and reduced cocaine intake and that it prevented cocaine-induced reinstatement following extinction. This highlights the potential therapeutic benefits of L-type Ca channel blockers in the management of cocaine use disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment:

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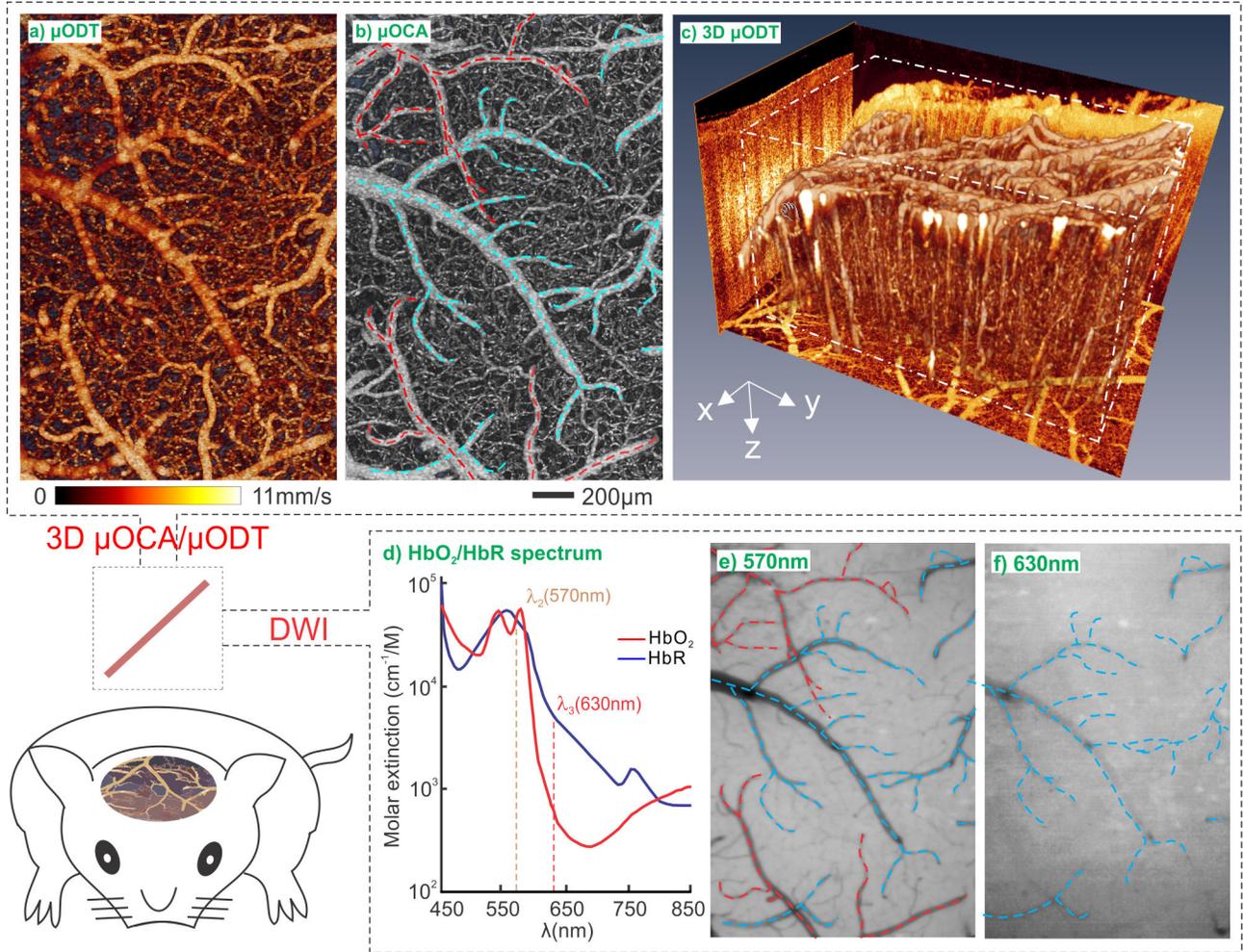
Fig. 1: Multimodal imaging platform for imaging cortical vascular and functional changes induced by cocaine in the rodent brain.

a) Quantitative cerebral blood flow velocity (CBFv) map of neurovascular network and **b)** its angiography and **c)** 3-dimensional (3D) CBF network imaging of cortex of a living animal obtained by ultrahigh-resolution optical coherence Doppler tomography (μODT) at λ_1=1.3μm. **d)** Absorption spectra of HbO2 and HbR to illustrate the principle of dual-wavelength optical imaging (DWI). The wavelength of 570nm and 630nm were selected in DWI to distinguish changes in HbO2 and HbR in cortical tissue. **e, f)** Spectral images of the cortex at λ2=570nm and λ3=630nm with separation of arteries (red arrows) and veins (blue arrows).
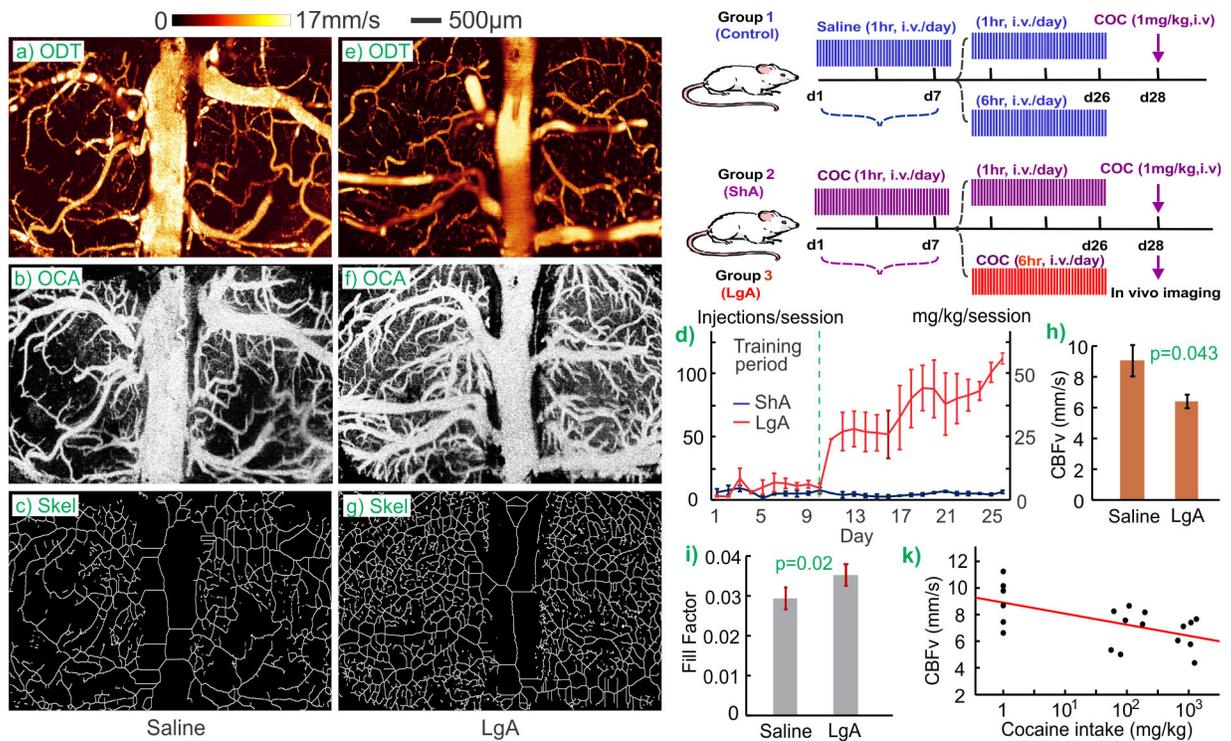
Fig. 2: Self-administration of cocaine decreased CBF but increased vascular density in prefrontal cortex (PFC).

Rat model for assessing the effects of long access (LgA) cocaine selfadministration on the prefrontal cortex (PFC) in **j)**. Rats self-administered cocaine (Groups 2 & 3) under a fixed-ratio-1 schedule (0.5mg/kg/injection) to compare with the controls (Group 1). **a,** Representative images of quantitative cerebral blood flow velocity (CBFv) of the PFC in control and LgA rats, respectively, illustrating the global CBFv decrease in the LgA animal. **b,** Representatives of vasculature angiographies of the PFC in control and LgA rats, and their skeletonized vasculature maps are presented in **c)** and **g)** illustrating the vascular density increase in the PFC of the LgA rat. **d)** increased drug intake in LgA during the escalation period (red curve, n=6) versus a stable drug intakes in ShA (blue curve, n=7). Left axis: number of injection per session, right axis: mg/kg/session. **h)** Significant CBFv decreases in CBFv in medium vessels ($\varphi=124.27\pm16.09$ μm) between control (n=6) and LgA (n=6) rats. **i)** Statistical comparisons of the fill factor (FF) of vasculature indicating that the vascular density was significantly increased in LgA compared to controls. **j)** Correlation of the mean CBFv with the total dose of cocaine intake (in log10 of mg/kg), indicating a moderately strong association (correlation coefficient r=−0.6, p=0.008) between the CBFv and the amount of cocaine administered to the animals. k). Correlation between CBFv and doses of cocaine intake (in $\log_{10}$ scale), indicating an inverse association (r=−0.6, p=0008) between CBFv and the amount of cocaine administered by the animals.
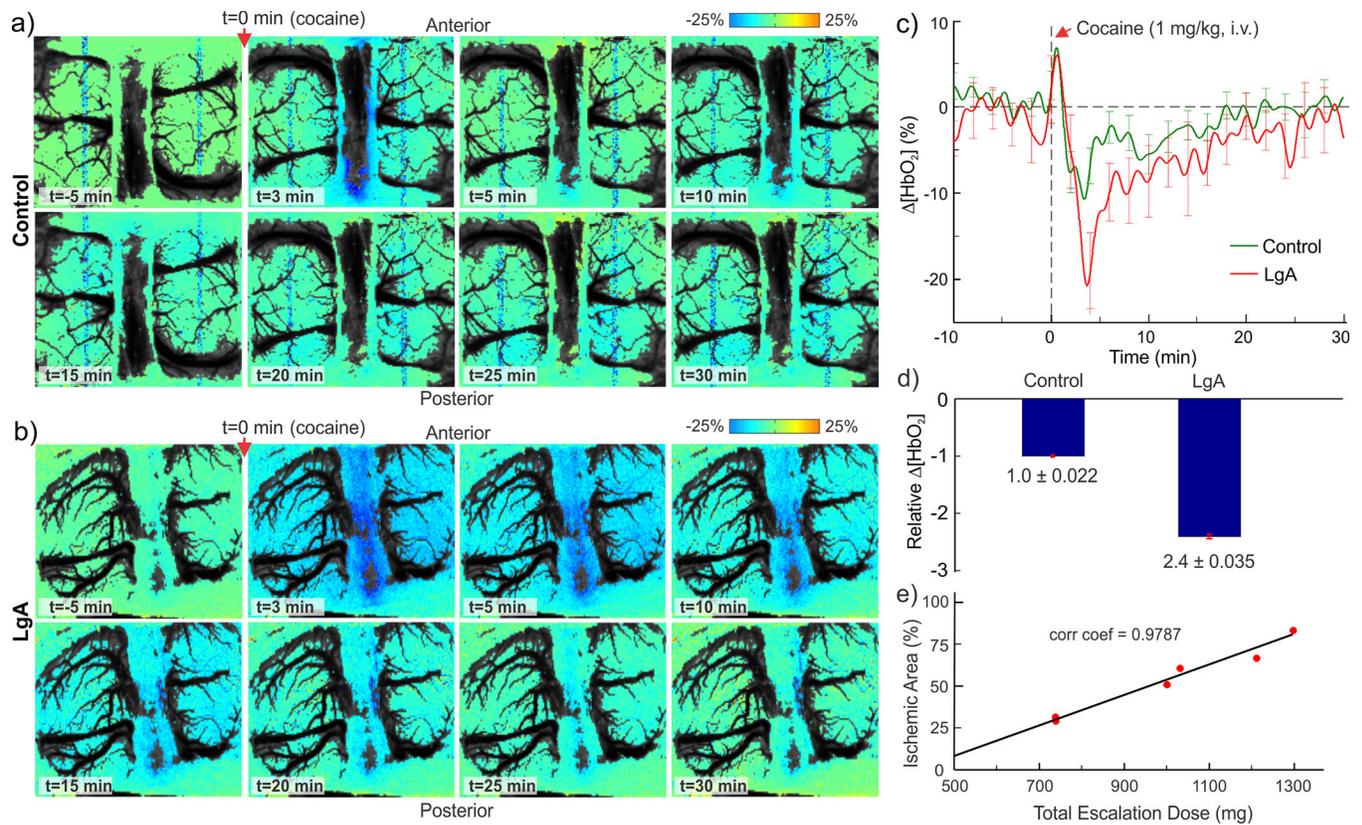
Fig. 3: Decreases in oxygenated hemoglobin (Δ[HbO2]) in the PFC with acute cocaine challenge are greater in LgA than in Control animals.

a) Representative full field [HbO2] map of the PFC before and after acute cocaine (1mg/kg, i.v.) in a cocaine-naïve (i.e., control) and LgA in **b**. **c)** Time course of mean [HbO2] changes in response to cocaine (1mg/kg) in control (n=6) and LgA (n=6). **d)** Total decrease of [HbO2] over the recording period (t=30 min) induced by cocaine in control and LgA rats (p=0.024). Cocaine induced a transient [HbO2] change (i.e., t< 5 min) in controls whereas the [HbO2] decrease in LgA animals was persistent (i.e., t 27 min) and observed across the whole FOV. The HbO2 reductions are consistent with 'ischemic' effects of cocaine in the LgA animals. **e)** Correlation of the 'ischemic' area (i.e., total pixels of Δ[HbO2] −10%) with the total escalation dose of cocaine in the LgA animals, indicating a strong association between the severity of the ischemic area and the doses of cocaine administered by the animals.
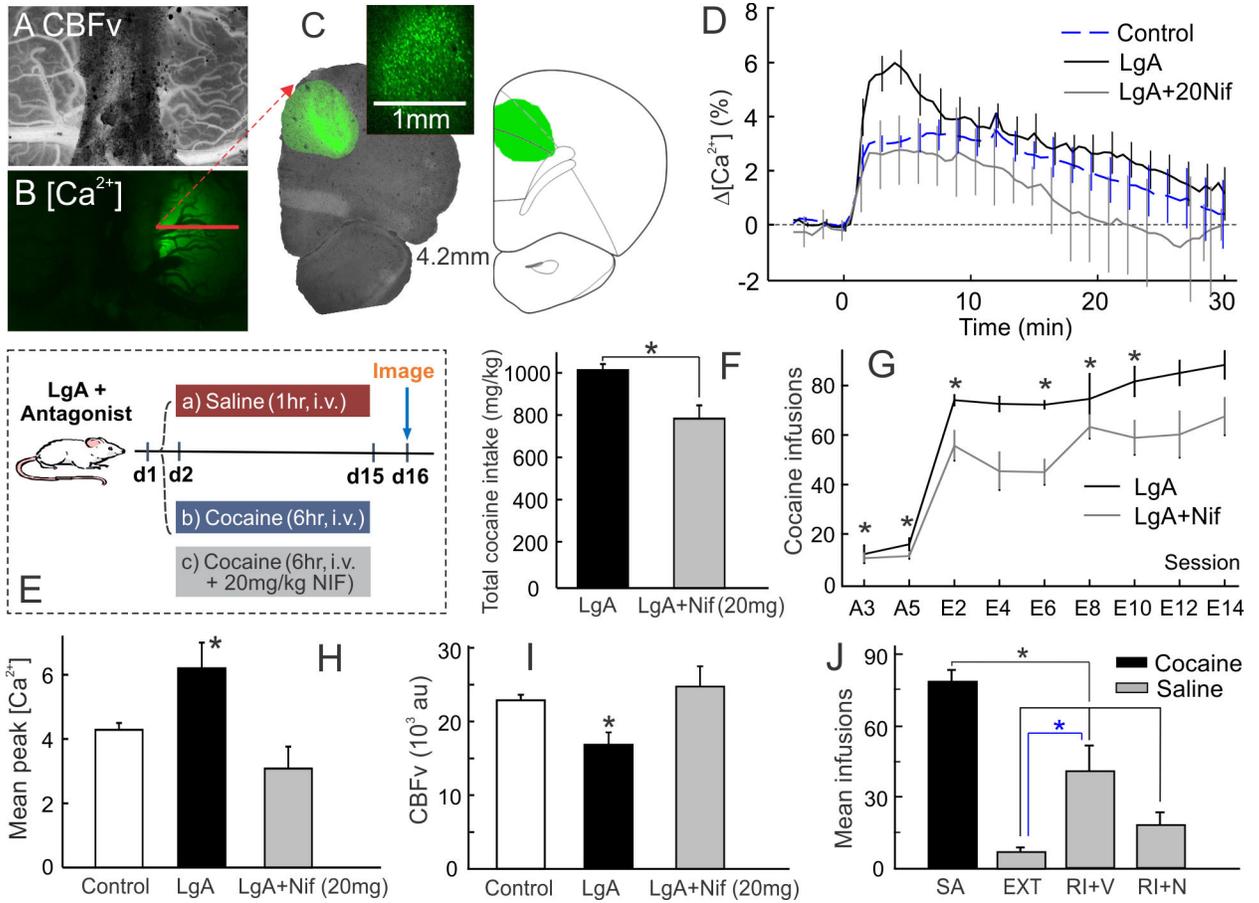
Fig. 4. Treatment with calcium antagonist during self-administration reduces drug taking and prevents reduction in CBFv.

A) shows a representative image of laser speckle (830nm) used to calculate CBFv. B) shows a representative Ca^{2+} fluorescence image. C) shows a cross-sectional representation of fluorescence. D) time course of calcium change following a cocaine (1mg/kg) injection at time 0, in animals with either history of cocaine or saline SA. E) shows the timeline for the nifedipine (NIF) SA experiment. **F)** Total cocaine intake by LgA and LgA+NIF rats over 14 days of self-administration; 20mg/kg NIF (i.p.) reduced cocaine intake supporting the hypothesis that reduction in CBFv contributes to escalation of intake. G) mean cocaine intake across sessions. H) peak fluorescence amplitude. LgA causes increased response to cocaine challenge which was blocked by nifedipine treatment. **I)** CBFv in rats with a history of saline, LgA or LgA+NIF; treatment with NIF blocked the reduction of CBFv caused by cocaine self-administration. J) test of reinstatement (n=8) last day of self-administration (SA), extinction (EXT), reinstatement following vehicle (RI+V), following 20 mg/kg nifedipine (RI+N). * indicates a significant difference (p<0.05).
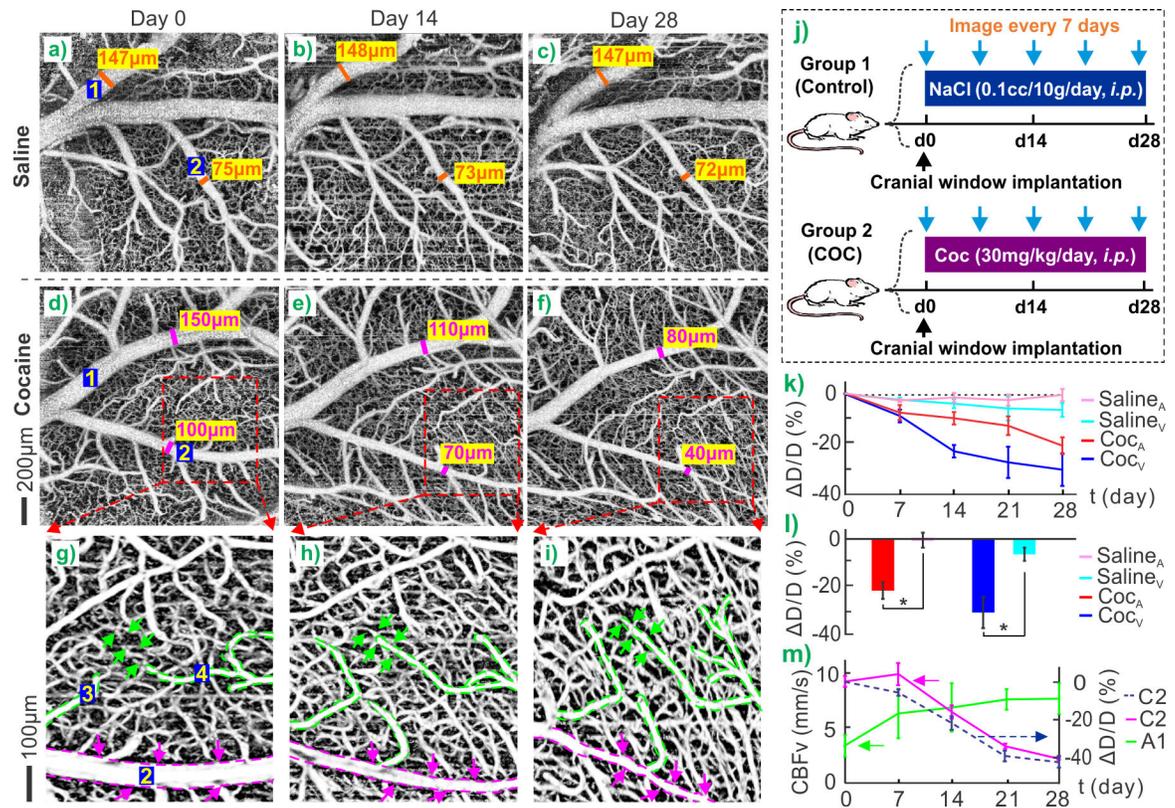
Fig. 5: Longitudinal imaging of chronic-cocaine elicited vasoconstriction and angiogenesis in the prefrontal cortex (PFC) of mice.

Mouse model for longitudinal imaging of cocaine-induced changes in neurovascular networks in the PFC **(j)**. Mice were divided into two groups, i.e., naïve (control) group and cocaine group. **a-c)** μOCA images of the PFC from a control mouse at baseline (i.e., 0-day) and at 14th day and 28th day of saline pretreatment (0.1cc/10g/day, i.p.), showing that there were no changes in vessel diameters as a function of time. **d-f)** μOCA images of the PFC from a mouse at baseline and at 14th and 28th day of daily cocaine exposures (30mg/kg/day, i.p.), showing vasoconstriction of blood vessels (e.g., the diameters of vessel '1' and '2' decreased). **k)** Time courses of vascular diameter changes in controls (n=5) and cocaine exposed animals (n=7). The diameters of both arteries and veins progressively decreased as a function of days of cocaine exposure, indicating an exacerbation of vasoconstriction with more extended exposures. **l)** Comparisons of vascular diameter changes at 28 days between cocaine treated and the control mice; cocaine mice showed greater reductions in both veins and arteries. **g-i)** 'Zoom-in' visualization of cocaine-induced angiogenesis surrounding a constricted vessel as a function of time, e.g., Day 0, 14 and 28 of cocaine treatments. **m)** Time course of changes in diameter (dashed blue curve) and CBFv (pink curve) in the constricted vessels (e.g., pink tracks) and growing angiogenesis (e.g., green tracks) shown in g-i. It indicates that, while the constricted vessels reduced their diameter and CBFv, the CBFv angiogenesis in the areas was initialized and gradually increased. *: p<0.05 difference from control animals.
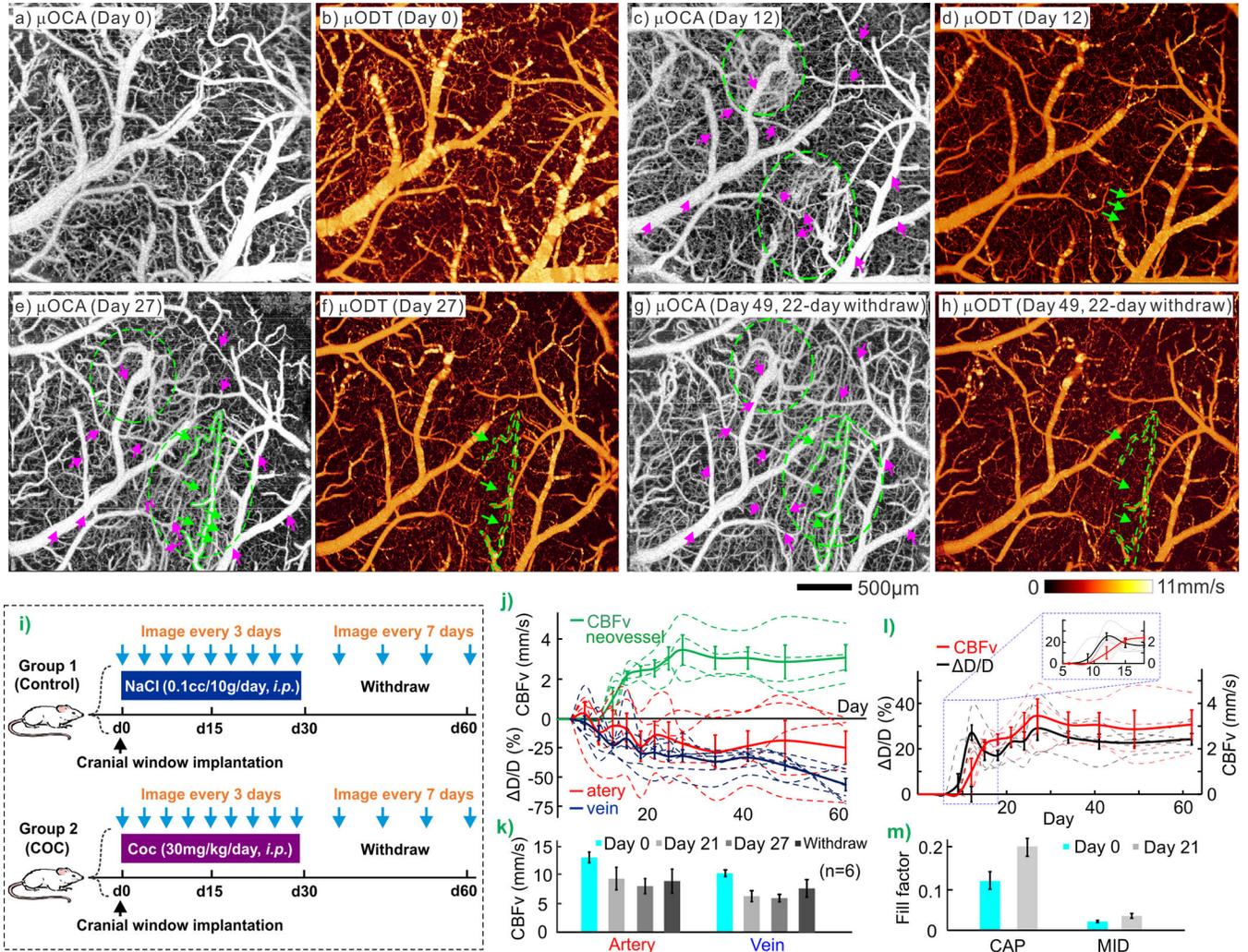
Fig. 6: Longitudinal ultra-high resolution μOCA and μODT to identify chronic-cocaine elicited vasoconstriction and angiogenesis in somatosensory cortex.

a, c, e, g) μOCA images of mouse cortex before cocaine (i.e., Day 0) and after cocaine exposures of 12 and 27 days (30 mg/kg/day i.p) and 22 days withdraw from the last does of cocaine exposure; **b, d, f, h)** Corresponding μODT images simultaneously recorded with μOCA to monitor CBFv changes with neurovascular network, indicating the chronic cocaine elicited vasoconstriction (e.g., pink arrows in c, e, g) and the corresponding microvascular adaptation (CBF reduction illustrated in d,f,h to compare with baseline in b, and micro-neoangiogenesis, green circles). Interestingly, although neoangiogenesis occurred on Day 12 as shown in c), little detectable flows were observed in these vessels as shown in d) (e.g., neovessel pointed by green circles in c and green arrows in d). Image size: 2.0×1.5×1.0 mm3. **i)** Longitudinal imaging of cocaine-induced changes in neurovascular networks in the mouse somatosensory cortex and effects of 35 days of withdrawal. Mice were pretreated with saline (~0.1cc/10g/day, i.p.) or cocaine (~30mg/kg/day, i.p.) for 30 days followed by 30 days of withdrawal. μOCA and μODT imaging were done once every 3 days until 28 days in the pretreatment period, and then reduced to once every 7 days (i.e., each week) during the withdrawal period until the end of the experiment (i.e., ~60 days). **j)** Time course of blood

flow (CBFv) changes in individual arteries (red dashed lines) and veins (blue dashed lines) and CBFv development in angiogenesis (green dashed lines). Their mean changes are presented as solid lines correspondingly. **k)** Statistical results of CBFv changes in arteries and veins after 21 days of cocaine treatment with respect to the baseline (day 0). It shows the CBFv decreased from 13.07±0.92 mm/s to 9.40±1.95 mm/s in the arteries (n=6) and from 10.32±0.56 mm/s to 6.36±0.94 mm/s in the veins (n=6), corresponding to mean CBFv reductions of 28.08% in arteries and 38.42% in veins. From last day of cocaine treatment (Day 27) to the withdrawal of 25±5.4 days, the CBFv in arteries were 8.08±1.29mm/s and 9.01±2.09mm/s respectively (p=0.879>0.05), and in veins 6.02±0.63m/s and 7.74±1.52mm/s, respectively (p=0.983>0.05). **l)** changes in diameters and blood flow in new microvessels. It shows that the vessel diameter peaked prior to their CBFv peak, which indicates angiogenesis development; **h)** Statistical results of vascular density changes in capillary bed and middle size vessels after 21 days of chronic cocaine compared with their baseline (0-day). It shows the vascular density increased from 0.12±0.02 to 0.20±0.02 in capillaries (fill factor) (p=0.029) and from 0.02±0.002 to 0.03±0.01 (p=0.026) in middle vessels.