Supplementary File 1

1. Detailed Materials and Methods

1.1 Subjects and Procedures

Subjects in this study were recruited for a study of a phenotyping battery that was designed to provide behavioral and brain imaging phenotypes for future OUD medication development research. An initial phone interview screened potential participants for study inclusion. Subjects who passed the phone screening were invited for a more thorough in-person screening interview (visit 1) during which all study procedures were explained and written informed consent was obtained. A medical history and physical examination were conducted by a licensed physician or nurse practitioner under supervision of a physician. Psychiatric and substance use histories were conducted using the Mini International Neuropsychiatric Interview (MINI) for Diagnostic and Statistical Manual version 5 (DSM-5) (American Psychiatric Association, 2013), including a review of medications/treatment. The MINI (https://harmresearch.org/index.php/product/mini-international-neuropsychiatric-interview-mini-7-0-2-4/) is a short, structured diagnostic interview for the DSM that generates DSM-5 diagnoses, including opioid use disorder (OUD) severity (Mild, Moderate, Severe). Blood chemistries, complete blood count, and urinalysis were obtained from each subject. The results of the MINI interview were presented to and confirmed by a physician co-author (JLS or FGM) who is dual board-certified in psychiatry and addiction medicine. All diagnoses were determined prior to any fMRI analysis. For the OUD group, inclusion criteria were DSM-5 diagnosed OUD and age between 18 and 70 years. Exclusion criteria were any history of schizophrenia, seizure disorder, significant head trauma, any changes to psychoactive medications within 30 days of the study period, any other DSM-5 diagnosed Substance Use Disorder with a severity diagnosis greater than the subject's OUD severity, or DSM-5

diagnosed severe Alcohol Use Disorder. For the HC group, the only inclusion criterion was age 18 to 70. Exclusion criteria were any history of substance use disorder, any history of schizophrenia, seizure disorder, significant head trauma, or any changes to psychoactive medications within 30 days of the study period.

Subjects who qualified for the study completed three additional visits: a visit in which they completed study behavioral measures and questionnaires (visit 2), a visit in which they completed safety screening for the MRI scan and a mock MRI session (visit 3), and a visit in which they completed an MRI scanning session (visit 4). All the fMRI scans in this study were resting-state fMRI scans, and thus none of the fMRI scans in this study involved tasks inside the scanner. The timing of the visits was as follows: after the initial in-person screening visit, the behavioral assessment visit was scheduled to take place within about 14 days (1-2 weeks). The MRI screening and mock scan visit typically occurred within 1-3 weeks after behavioral assessment. The MRI scan occurred within 30 days of the MRI screening visit. Participants were asked to refrain from smoking 1 hour and drinking caffeine 3 hours before their MRI scan. Urine drug screens (UDS) and breath alcohol screens were collected at each visit. A clinical assessment by a physician or nurse practitioner was performed during the MRI visit before scanning to ensure that subjects did not meet DSM-5 criteria for drug intoxication at the time of the scan. Subjects were also assessed for gross signs of drug withdrawal by a physician or nurse practitioner during the MRI visit before scanning. None of the UDS on the day of scanning were positive for HC subjects. None of the breath alcohol screens on the day of scanning were positive from HC or OUD subjects.

80 subjects (33 OUD, 47 HC) completed the MRI scanning session. In our final analyses, we only included subjects who met strict criteria for head motion (Parkes et al., 2018), had all physiological data needed to correct for physiological noise, and for OUD subjects had at least one UDS positive for

either illicit opioids or buprenorphine or methadone. This initial refined group included 25 OUD subjects and 39 HC subjects. We then further refined our HC sample to match more closely the age and sex composition of the OUD group until there was an equal number of subjects in both groups. The authors of the FMRIB Software Library (FSL) method, MELODIC (Multivariate Exploratory Linear Optimized Decomposition into Independent Components; Beckmann & Smith, 2004), which we used to generate group ICA network components, recommend balancing the number of subjects in each group. According to the authors of MELODIC, unequal group size can result in the group-ICA components being more heavily influenced by the larger group, which can result in lower sensitivity to detect differences in brain regions that differ between the groups (Bijsterbosch et al., 2017, p. 67). Furthermore, "… it is generally preferable to avoid having unequal group sizes to start with" (Bijsterbosch et al., 2017, p. 67). As an additional benefit of dropping healthy control subjects to achieve an equal number of subjects in both groups (25 subjects per group), this enabled us to match the two groups more closely for age and sex. It should be noted that all balancing of the subject groups and dropping of subjects had been done prior to any fMRI analysis, and that no OUD subjects had been dropped from this study to balance the number of subjects, age, or sex between groups. 25 OUD subjects (15 males and 10 females; proportion of males to females = 0.60) and 25 HC subjects (13 males and 12 females; proportion of males to females = 0.52) were included in the final analyses.

1.2 Behavioral Measures

NU scores were extracted from a short form of the UPPS-P scale (Lynam, 2013). The range of possible scores on the NU scale is 4 to 16. Scores for each of the UPPS-P components were also recorded. Opioid use was assessed by UDS (between 2 and 7 UDS collections for each subject; collected within 15 months before the MRI scan date). Tobacco use was assessed by the Fagerström Test of Nicotine

Dependence (Heatherton et al., 1991). All behavioral and demographic data were analyzed using JMP (JMP, Version 14. SAS Institute Inc., Cary, NC, 1989-2019).

A two-sample T-test was performed to test for statistical significance between groups with respect to age, NU, and all other sub-scores and the total score from the UPPS-P. An unequal variance two-sample T-test was performed for mean relative framewise displacement (mFD; a measure of head motion) because a Brown-Forsythe test determined the variances between groups were significantly different ($p<0.02$). The median and interquartile range are reported for time since last opioid use because time since last opioid use was not normally distributed. A Chi-Square test indicated the number of males and females did not significantly differ between the two groups ($X2=0.33$, df=1, $p=0.5$).

1.3 MRI Acquisition

MRI scans were acquired using the Philips Medical Systems (Best, Netherlands) Ingenia wide-bore dStream 3T MRI scanner, with a 32-channel receive head coil. Single shot gradient-echo echoplanar imaging (EPI) was used for acquiring fMRI data. The fMRI acquisition parameters were: parallel imaging SENSE in-plane acceleration factor 1.5, multiband factor 3, repetition time 1625 ms, echo time 30 ms, flip angle 52°, field of view 240 mm (anterior-to-posterior) × 240 mm (left-to-right) × 125.70 mm (foot-to-head), in-plane resolution 2.5 mm × 2.5 mm, 45 axial slices, slice thickness 2.5 mm, interslice gap 0.30 mm, 420 repetitions per run after 12 dummy acquisitions, and total duration was 11 minutes 22 seconds. Subjects completed the resting state fMRI scan with a black fixation cross on a white screen and with eyes open. Prior to the fMRI scan, two spin-echo echoplanar scans, using the same echo spacing and geometry as the main run, were acquired with opposite phase-encode directions to calculate distortion-correction. A T1-weighted 3-Dimensional Magnetization Prepared Rapid Gradient Echo (3D-MPRAGE) scan with acquisition voxel size = [1 x 1 x 1] mm and 160 axial

slices was acquired for offline co-registration with the fMRI scans, and a T2-weighted Fluid-Attenuated Inversion Recovery (FLAIR) scans was read by a neuroradiologist to screen for incidental pathology. Peripheral pulse rate (using an MRI compatible finger-clip pulse oximeter) was electronically recorded continuously during the fMRI scans for offline removal of artifactual physiological signals. Task-based fMRI scans were also acquired, for analysis for a different study than the present experiment but were acquired after the resting state fMRI scan. In some instances where the resting state scan had to be repeated, the repeat scan was acquired after the task-based scan.

1.4 MRI Preprocessing

Initial removal of signal outliers during each fMRI raw time series (run) was performed using AFNI's 3dDespike function (Cox 1996, Version 20.1.02). Within each voxel, a timepoint with fMRI signal greater or equal to 6 standard deviations from the mean of the run within that voxel was substituted with the average of the signal of the two nearest neighboring time points. Physiologic noise correction with heart rate as an input was implemented via AFNI's 3dretroicor (Cox 1996, Version 20.1.02; Glover et al., 2000), with adjustment for slice time acquisition differences. The PhysIO Toolbox (Kasper et al., 2017), implemented in SPM, was used to noise-correct the peripheral pulse waveforms and extract the timing of the peak pulse amplitudes before entering into AFNI's 3dretroicor. Slice timing correction of the fMRI signal was performed via SPM12 (http://www.fil.ion.ucl.ac.uk/spm/).

From the preliminary spin-echo scan pair with opposite phase-encode directions, susceptibility-induced off-resonance field correction was conducted using the method implemented in FSL "topup" software (www.fmrib.ox.ac.uk/fsl/).

Quality control for head motion was assessed via the FSL FEAT pre-stats module (www.fmrib.ox.ac.uk/fsl/), using stringent criteria based on Parkes et al. (2018), in which fMRI runs

were eliminated from further analysis if any of the following criteria were met: (1) if the mean relative framewise displacement (mFD) was greater than 0.20 mm; or (2) if the number of timepoints with suprathreshold framewise displacements (FDs) (defined as FD greater than 0.25 mm) was greater than 20% of the total number of volumes in the run; or (3) if any individual FD was greater than 5 mm; or (4) if the run contained less than 4 continuous minutes without any suprathreshold FDs. FD (sometimes called FD_{Jenk}) was calculated by the FSL MCFLIRT motion-correction program, as implemented in the FEAT pre-stats module, using the root mean squared volume-to-volume displacement of all brain voxels measured from the six head motion parameters (Jenkinson et al., 2002; Parkes et al., 2018). Relative FD was calculated relative to the FD of the preceding timepoint. Mean relative FD (i.e., mFD) is the average of relative FD values across the entire resting state scan for a participant. The FSL "FEAT" pre-stats module was conducted using rigid-body realignment of the fMRI timeseries, calculation of parameters for registration to the MPRAGE and subsequent parameters for linear and non-linear transformation to MNI standard space, spatial smoothing with a Gaussian kernel of 5 mm FWHM, and no highpass or lowpass filtering yet at this step.

The contents of the output folder from the previous FEAT pre-stats step served as the input to ICA-AROMA (Pruim et al., 2015a; Pruim et al., 2015b) for removal of head-motion related signal by using nonaggressive denoising applied to the spatially smoothed fMRI images on which ICA-AROMA used the spatial realignment and registration parameters from the previous FEAT step.

Removal of other artifactual signal from the ICA-AROMA-denoised fMRI timeseries using the aCompCor procedure as implemented in CONN software in Matlab (Behzadi et al., 2007; www.nitrc.org/projects/conn, RRID:SCR_009550; MATLAB 2019), generating 5 principal components from an unsmoothed white matter region of interest created within CONN with erosion settings: Binarization threshold = 0.99, absolute value; Exclusion mask = 0.005; Erosion level = 5;

Number of erosions = 2; Erosion neighborhood = 0. Also using CONN, 5 principal components were extracted from an unsmoothed twice eroded cerebrospinal fluid region of interest generated from FSL FAST tissue segmentation with 0.99 probability CSF and using the FSL MNI152_T1_2mm_VentricleMask. Subsequent multiple regression general linear model was used to remove these white matter and CSF components from the fMRI timeseries.

The FSL applywarp command was used to transform the ICA-AROMA-and-aCompCor-denoised fMRI timeseries into MNI space, based on the linear transformation and nonlinear warp parameters generated from the FEAT pre-stats step. High pass filtering was performed with a cutoff of 0.008 Hz (125 s). Low pass filtering was not performed as studies have suggested that relevant functional connectivity signal may be lost by doing so (Boubela et al., 2013; Chen & Glover, 2015).

1.5 Functional Connectivity Analysis

Group independent component analysis (group-ICA) maps were created via FSL MELODIC (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/MELODIC; Beckmann & Smith, 2004), in which the preprocessed fMRI scans from all 25 OUD subjects and all 25 HC subjects were temporally concatenated as input. The output maps of FSL MELODIC group-ICA were visually inspected with a threshold of $Z \geq 4$ (which was calculated by dividing the original component connectivity strength at each voxel by the standard deviation of the residual noise) to determine which components most closely mapped the DMN, SN, left ECN (LECN), and right ECN (RECN), compared to visual inspection of maps of those networks generated from previous studies (Menon, 2011; Shirer et al., 2012; Sridharan et al., 2008). Group-ICA outputs with components of 15, 20, 25, and 30 were selected for comparison based on the number of components chosen in previous studies (Kuo et al., 2019; Li et al., 2015; Li et al., 2018). The number of components was set at 30 because it gave the best visual representation of the DMN, SN, LECN, and RECN from the current dataset.

Dual regression analysis was then performed in FSL (Nickerson et al., 2017; https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/DualRegression) to obtain subject-specific component maps. The FSL technique of Group Independent Component Analysis (GICA) (Bijsterbosch et al., 2017, p. 59) derives network maps that are common to all the subjects (i.e., fMRI data from all subjects in both groups combined are included in the GICA). After the GICA procedure is complete, stage 1 of the FSL dual regression (Nickerson et al., 2017) uses the GICA generated network maps to estimate a subject-specific time-course (timeseries) for each network. Each subject-specific time-course essentially reflects the average time-course across voxels in the corresponding network map (after taking into account the contributions of the other networks) (Nickerson et al., 2017). Those subject-specific time-courses were then normalized by their amplitude (represented by the standard deviation of the time-course) to allow for measurement of network-wide and localized signal amplitude differences, in addition to differences in the spatial distribution of connectivity strength across subjects (Nickerson et al., 2017). In stage 2 of dual regression, the subject-specific time-course for each network is then used as a template to generate a subject-specific spatial map for that network. Specifically, in stage 2 of dual regression, a linear regression analysis is conducted within each voxel, where the dependent variable is the individual subject's observed fMRI time-course within that voxel, and the regressor is the subject-specific time-course which is characteristic of the entire network. This regression analysis estimates a regression coefficient, which essentially scales the fit of the observed voxel time-course to the network template time-course (Nickerson et al., 2017). The parameter estimate (beta value) of the regression coefficient at each voxel constitutes the subject-specific spatial map of relative functional connectivity (Bijsterbosch et al., 2017, pp. 63-64) across the network. Because of the variance normalization procedure used in the dual regression analysis, the map of regression coefficients obtained from stage 2 represents the relative functional connectivity across the voxels in the network, and also the relative magnitude of the BOLD signal at each voxel (Nickerson et al., 2017). The

amplitude of the resting fMRI signal is believed to reflect an important aspect of functional connectivity (reviewed in Nickerson et al., 2017).

Non-parametric permutation tests were then performed via the Permutation Analysis of Linear Models (PALM) program (Winkler et al., 2014; https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/PALM) using the subject-specific spatial maps of parameter estimates output of stage 2 of the dual regression to compare the within-network functional connectivities (i.e., spatial maps of parameter estimates) of the DMN, SN, LECN, and RECN between groups. The FSL standard Threshold Free Cluster Enhancement (TFCE) was used to identify statistically significant clusters of voxels while maintaining family-wise-error (FWE) control (Smith & Nichols, 2009). PALM computed FWE correction for the number of voxels and for the number of networks, 4, that were input, and also for the number of contrasts, 2, within each model. The FWE correction of the 2 contrasts within each model accounted for two-tailed significance (OUD>HC and HC>OUD for group functional connectivity differences and positive and negative regression slopes for the regression of functional connectivity on NU) (Alberton, et al. 2020). FWE correction was used because there may be issues with using FDR correction in functional network analysis, in terms of possibly not preserving the spatial relationship among voxels within a given network (Winkler et al., 2016). According to the authors of the PALM program (Winkler et al., 2016), FDR multiple comparisons correction may not guarantee that the spatial relationship among voxels within a given test is preserved when applied across multiple tests. Issues may arise when correcting across multiple ICA-identified networks (which we performed in our study) while maintaining FDR control across voxels within a given network. These issues are mitigated when using full FWE correction as implemented in the PALM software (Winkler et al., 2016). FWE correction for multiple comparisons also has the advantage of being more stringent than FDR correction (Bijsterbosch et al., 2017, p. 75).

In addition to the whole-network ICA analysis, this study also assessed a priori specific ROIs within the SN (left anterior insula, right anterior insula, and dorsal anterior cingulate cortex (dACC)), and a priori specific ROIs within the DMN (posterior cingulate cortex (PCC) and medial prefrontal cortex (mPFC)), as these regions have been proposed to be key nodes within those networks (Menon, 2011; Zhang & Volkow, 2019). The anterior insula and dACC are in the SN and have both been associated with facilitating switching between the DMN and ECN based on the internal or external nature of the stimuli, respectively (Kerns et al., 2004; Sridharan et al., 2008; Menon & Uddin, 2010). The mPFC and PCC are in the DMN and have been associated with processing and attributing meaning to personally relevant stimuli (Andrews-Hanna et al., 2014). We hypothesized that the functional connectivity of the left anterior insula, right anterior insula, and dACC regions of interest within the SN and the PCC and mPFC regions of interest within the DMN, would be weaker in OUD compared to HC. We compared the mean connectivity strength parameter estimates between groups within each of the a priori ROIs (left insula, right insula, and dACC in the SN; dmPFC and PCC in the DMN). Region of interest (ROI) boundaries (for the PCC and mPFC of the DMN and the left anterior insula, right anterior insula, and dACC of the SN) for another set of exploratory analyses were set by creating a mask for each region based on functional network templates obtained from the Stanford FIND lab website (http://findlab.stanford.edu/functional_ROIs.html), that were reported in Shirer et al. (2012). Mean connectivity strength parameter estimate values (output from stage 2 of dual regression) were calculated for each ROI within their respective networks and were used as the input for a nonparametric analysis (following group-mean centering) performed using PALM comparing the two groups. PALM outputs were FWE corrected for multiple comparisons for the 5 ROI inputs as well as the 2 contrasts for each ROI (OUD>HC and HC>OUD).

To test for associations between hypothesized functional connectivities and behavioral data, PALM was used to perform a voxel-wise regression analysis of the subject-specific SN functional connectivity on the mean-centered NU scores for both subject groups testing for both main and group interaction effects of NU. We first tested for group x NU interaction effects, and then assessed the main effects of NU if there were no statistically significant group x NU interaction effects (FWE-corrected for voxels $p<0.05$) per the recommendation of the authors of the FSL neuroimaging analysis software that we used (General Linear Model for neuroimaging guide https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/GLM) as well as Kutner et al. (2005; pp. 306-308, 312-313, 326-327, 921-925, 932-933). For exploratory analyses, using the same procedure, the regressions of the within-network functional connectivities of the DMN, LECN, and RECN on mean-centered NU scores were computed. For the analysis of the effects of mFD on functional connectivity and post-hoc analysis of time since last opioid use, the same procedure was followed. Once significant group interaction effects were ruled out, the regressions of the within-network functional connectivity of the DMN, SN, LECN, and RECN on mean-centered mFD and hours since last opioid use were computed.

The anatomical location of the only significant cluster reported in section 3.2 of the manuscript was determined by visually inspecting the location of the cluster overlaid on the Harvard-Oxford Cortical Structural Atlas in FSLeyes (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/Atlases) and converting the MNI coordinates of the anterior-posterior and dorsal-ventral borders of the cluster into Talairach coordinates using a publicly available widget (https://bioimagesuiteweb.github.io/webapp/mni2tal.html; Yale BioImage Suite Package - MNI to Talairach Mapping; Lacadie et al., 2008). Then, we compared those Talairach coordinates to the dorsolateral prefrontal cortex Talairach coordinates given in the histological study by Rajkowska & Goldman-Rakic (1995).

In order to examine heterogeneity of our OUD sample, we also performed two post-hoc analyses. In the first analysis, we compared OUD subjects with at least one UDS positive for buprenorphine or methadone to OUD subjects with UDS positive for only illicit opioids. In the second analysis, we regressed the subject-specific component maps onto mean-centered self-reported time since last opioid use, measured in hours.

We had planned to perform an ANCOVA with NU as a covariate if the main effect of NU in the preregistered regression analysis of functional connectivity on NU was statistically significant, but the regression results were not statistically significant. Based on recommendations from a standard statistics textbook (Kutner et al., 2005, pp 347, 919, 940), for a concomitant variable to be included as a covariate in ANCOVA, there should be a statistically significant regression relationship of the concomitant variable with the response variable. If potential covariates have no relation to the response variable, then nothing is to be gained by including them in ANCOVA (Kutner et al., 2005, p. 919), and in our case, the reduction in the degrees of freedom from adding such covariates to the model may be detrimental, given the relatively small sample size in our study. Furthermore, a worsening of the model's performance can occur when variables are kept in the model that are not related to the response variable, or if there is no regression relationship to allow for extrapolation of the regression line of the covariate between the means of the two groups (Kutner et al., 2005, pp. 347, 940). None of the NU regression results for any of the networks were statistically significant. We also performed a regression analysis of functional connectivity on head motion (mFD) and education for all four networks examined to determine whether to include head motion or education as a covariate in ANCOVA. None of the head motion or education regression results for any of the networks were statistically significant and therefore mFD and education were not included as covariates in ANCOVA. Given that tobacco

use was imbalanced between groups, we compared the functional connectivity of tobacco using OUD to non-tobacco using OUD to investigate the effects of tobacco use on functional connectivity.

1.6 ROI Group Differences

OUD and HC did not significantly differ in mean connectivity strength for any of the 5 ROIs (p greater than 0.333).

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