

Brain Functional Connectivity Mapping of Behavioral Flexibility in Rhesus Monkeys

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The predisposition to engage in autonomous habitual behaviors has been associated with behavioral disorders, such as obsessive-compulsive disorder and addiction. Attentional set-shifting tasks (ASSTs), which incorporate changes governing the association of discriminative stimuli with contingent reinforcement, are commonly used to measure underlying processes of cognitive/behavioral flexibility. The purpose of this study was to identify primate brain networks that mediate trait-like deficits in ASST performance using resting-state fMRI. A self-pacing ASST was administered to three cohorts of rhesus monkeys (total $n = 35$, 18 female). Increased performance over 30 consecutive sessions segregated the monkeys into two populations, termed High Performers (HP, $n = 17$) and Low Performers (LP, $n = 17$), with one anomaly. Compared with LPs, HPs had higher rates of improving performance over sessions and completed the 8 sets/sessions with fewer errors. LP monkeys, on the other hand, spent most of each session in the first set and often did not acquire the first reversal. A whole-brain independent components analysis of resting-state fMRI under isoflurane identified four strong networks. Of these, a dual regression analysis revealed that a designated “executive control network,” differed between HPs and LPs. Specific areas of connectivity in the rhesus executive control network, including frontal cortices (ventrolateral, ventromedial, and orbital) and the dorsal striatum (caudate, putamen) correlated with perseverative errors and response latency. Overall, the results identify trait-like characteristics of behavioral flexibility that are associated with correlated brain activity involving specific nuclei of frontostriatal networks.

Key words: attentional set-shifting; frontostriatal circuits; resting state networks; rhesus monkey

Significance Statement

Resting state functional connectivity MRI in rhesus monkeys identified specific nuclei in frontostriatal circuitry that were associated with population differences in perseverative and impulsive aspects of cognitive flexibility.

Introduction

A vital function of the primate brain is to plan and execute complex sequences of behavior for optimal task-performance as well as to recognize and alter action selection when contingencies change (McDaniel and Kearney, 1984; Scott, 2002). In general, action selection requires cognitive processes that provide efficient flexibility in using learned associations in the context of

changing environments. Individuals differ in their capacity to integrate these cognitive processes, which include attention to stimulus salience, working memory, shifting attention, inhibiting learned responses, and the decision to execute the response (Dajani and Uddin, 2015; Friedman and Miyake, 2017; Tait et al., 2018). Deficits in behavioral flexibility manifest as a reliance on automatized habitual behavior as is prevalent in obsessive compulsive and addictive disorders, schizophrenia, and early stages of Parkinson's disease (Gillan and Robbins, 2014; Culbreth et al., 2016). Improved understanding of the neurobiological bases of processes that underlie behavioral flexibility is needed for identifying risk, diagnosis, and evaluation of treatment efficacy for behavioral disorders.

Attentional set-shifting tasks (ASSTs) provide measures of cognitive flexibility using serial discriminations of stimuli presentations (Tait et al., 2018). In general, ASSTs are highly translational across species and have been used in explorations of underlying brain activity (Dajani and Uddin, 2015; Morris et al., 2016). We developed

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an ASST that tests all monkeys within a cohort simultaneously using imbedded touchscreens in each monkey's home cage. The ASST does not involve training criteria before testing to capture predispositions to either advance on the ASST in a model-based fashion or respond in a random fashion with respect to acquiring the serial discriminations (Shnitko et al., 2017). To assess learning, a performance index (PI) composed of three independent measures (errors per trial, the final set reached, and the session duration) is calculated for each monkey over 30 sessions (Shnitko et al., 2017). The rate of the PI increase over 30 sessions (i.e., the learning curve) identified preexisting inflexible behavior as a trait that predicted habitual heavy alcohol drinking patterns (Shnitko et al., 2019), a finding that was replicated within and between cohorts of monkeys (Grant et al., 2021). The robust and reliable ASST prediction of habitual drinking suggested that performance on this ASST reflects a neurobiological trait and prompted the current study of living brain circuitry that may predispose individuals to engage in either flexible or habitual behaviors.

Anatomically separate, cortical-striatal circuitry loops are thought to subserve flexible versus habitual behaviors. Briefly, lesion and tract tracing studies helped identify two distinct circuitry loops. The associative circuit containing the prefrontal, entorhinal, and posterior parietal cortical projections to the primate caudate (or rodent dorsomedial striatum), and is proposed to mediate flexible changes to a behavioral pattern if reinforcement contingencies are changed; likewise, a sensorimotor circuit containing motor, somatosensory, and supplemental motor cortical projects to the putamen (or rodent dorsolateral striatum) is proposed to mediate habitual behaviors (Kelly and Strick, 2004; Yin et al., 2005, 2006; Gunaydin and Kreitzer, 2016; Heilbronner et al., 2016; Bostan and Strick, 2018). Therefore, individuals that have a bias in action selection using predominantly PFC and caudate circuitry are predicted to display flexible behaviors, whereas individuals with a bias toward engaging sensorimotor cortex and putamen circuitry activity will have a tendency toward habitual behaviors.

To test this hypothesis in the living brain, a useful approach is resting-state functional connectivity measured with MRI (rsfMRI). This technique has identified neural circuitry networks, including cortical-striatal connectivity, in behavioral flexibility assessed with ASST in human subjects (Seeley et al., 2007; Trick et al., 2014; Dajani and Uddin, 2015; Morris et al., 2016; Langley et al., 2021; Uddin, 2021). To date, identifying resting state networks (RSNs) using independent components analysis (ICA) in rhesus monkeys has relied on groups of relatively small subject size ($n \leq 6$) with few studies showing RSN alignment with cognitive measures (Lopez-Persem et al., 2020). Further, robust RSNs require a relatively large number of subjects, particularly if inferences of RSN substructure and behavioral outcomes are explored. Therefore, the following study addressed *in vivo* brain network connectivity and ASST performance in a large population of rhesus monkeys ($n = 35$) to test whether a comparison of RSNs between High Performers (HPs) and Low Performers (LPs) monkeys could identify brain regions linked to cognitive flexibility in the nonhuman primate (NHP) brain.

Materials and Methods

Animals

Rhesus monkeys (*Macaca mulatta*; $N = 35$, 18 females) were obtained from the Oregon National Primate Research Center (ONPRC) breeding colony. The monkeys were tested in three cohorts. Subjects were assigned to the study at 3.5–5.6 years of age and group-housed indoors in rooms with controlled temperature (20°C–22°C), humidity (65%), and

an 11 h light cycle. Individual housing cages ($0.8 \times 0.8 \times 0.9$ m) allowed a side-wall removal for social interaction (1–2 h per day). Each cage was equipped with an interactive panel that provided access to food and water, an LCD 11×13.25 inch monitor (Dell, Model E1715S) with overlaying touch-screen (Keytec, Model OPTIR Touch PPMT), a food receptacle connected to a 1 g pellet dispenser (Med Associates), and an infrared beam-manipulator below the receptacle. All touch-screen inputs and task-dependent outputs were controlled by a computer system using customized software (LabView 2011, SP1, National Instruments). Animals were acclimated to use the panel to obtain food and water as well as become familiarized with the monitor/touch-screen. The monkeys were not food-deprived, but the ASST was conducted in the morning before the first meal. The daily diet consisted of nutritionally complete banana-flavored 1 g pellets and fresh fruit with *ad libitum* access to water. All procedures were conducted according to the *Guide for the care and use of laboratory animals* (National Research Council, 2011) and approved by the Institutional Animal Care and Use Committee.

Brain imaging data acquisition

rsfMRI data were obtained at the ONPRC MRI Core Facility using a 3 T Siemens Magnetom Prisma MRI system equipped with 16 channel “pediatric” head-coil. Monkeys were anesthetized with ketamine (15 g/kg, i.m.) for transferring to the ONPRC MRI Core Facility, intubated and maintained on 1%–1.5% isoflurane throughout the imaging procedure. The rsfMRI protocol included EPI with TR/TE = 2290/25 ms, $N = 784$ slices, voxel size 1.5 mm isotropic, flip angle 79°, FOV = 96 mm. Whole-brain high-resolution T_2 -weighted structural scans using the “sampling perfection with application-optimized contrasts using different flip angle evolution” pulse sequence (TR: 3200 ms; TE: 385 ms; voxel size: 0.5 mm isotropic, FOV = 160 mm, $N = 672$ slices) were used to coregister the resting-state and anatomic data.

Brain imaging data preprocessing

T_2 -weighted images were averaged within a subject after rigid-body registration using “antsRegistrationSyN.sh” (Avants et al., 2008). The intensity bias field of each average image was corrected using a B-spline approximation routine and a hierarchical optimization scheme implemented by “N4BiasFieldCorrection” (Tustison et al., 2010). The corrected and averaged individual head-images were nonlinearly registered to the macaque brain ONPRC18 head-image (Weiss et al., 2021) using “antsRegistrationSyN.sh.” With the resulting transformation parameters, the ONPRC18 brain mask was reversely aligned to each subject image to generate the individual brain masks using a nearest neighbor interpolation method. The brain masks were used to extract each monkey brain followed by its registration to the common space of the ONPRC18 template. Preprocessing of rsfMRI data was conducted using Analysis of Functional Neuroimages (AFNI) software (<https://afni.nimh.nih.gov>). The afni_proc.py generated script for the preprocessing performed in this study is provided in Extended Data Figure 4-1. We regressed CSF and white matter signals (Jo et al., 2010).

rsfMRI independent component analysis

Group-level connectivity networks were derived using an ICA on the preprocessed rsfMRI data (Beckmann and Smith, 2004) using Multivariate Exploratory Linear Optimized Decomposition into Independent Components (MELODIC) software (<https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/MELODIC>). The number of components for the model was automatically estimated by MELODIC (Beckmann et al., 2005); and of the 10 independent components (ICs) identified with MELODIC in this study, 4 (signal-ICs) exhibited properties associated with previously described RSNs (Beckmann et al., 2005), overlapping primarily gray matter structures, exhibiting spatial distributions throughout the brain, and possessing low-frequency spectral power (Griffanti et al., 2017). The six remaining ICs were identified as noise-ICs based on the criteria above and shown in Figure 5.

Cognitive assessment

ASST training. The present version of ASST was modified from the Cambridge Automated Neuropsychological Test Automated Battery,

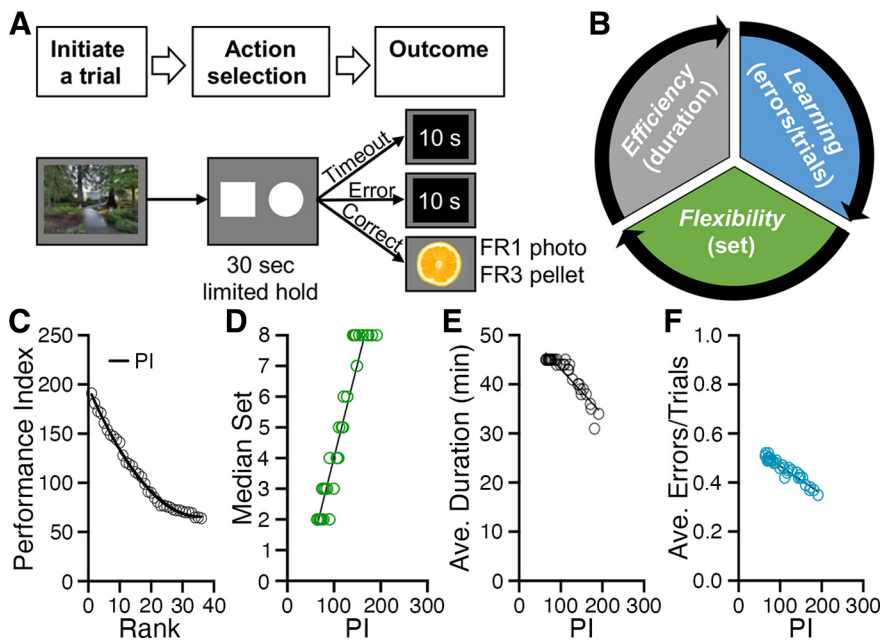


Figure 1. Ranking individual differences in the ASST with performance index. **A**, Schematic representation of a single trial within the ASST. Monkeys initiated a trial by touching a nature photograph, which was followed by a presentation of two geometrical shapes (both either black or white color during simple stimuli discrimination sets) or two geometrical shapes of different colors (sets with compound stimuli discrimination sets). The shapes remained on the screen for 30 s or until one of the shapes was touched. If the animal did not touch a shape in 30 s or touched a shape that was preassigned as “incorrect,” then a black screen appeared for 10 s and a new trial began. If the shape that was preassigned as “correct” was touched, then a photograph from a set of preferred images appeared for 2 s on FR1 schedule along with a banana-flavored pellet after each third correct response (FR3). This second-order schedule of reinforcement (FR1:FR3) was used to maintain consistent engagement in the task for the duration of the session (i.e., 45 min or until all 8 sets are completed). **B**, Cognitive functions assessed in the task performance: associative learning, attentional shifting, and efficiency. **C**, Individual differences in rank-ordered PI. The PI is a summation of three components measured in the ASST (shown in **D–F**) each scaled from 0 to 100 for a possible PI of 300 (see Materials and Methods). Shown here is the median PI across the 30 sessions for each monkey. Note the inflection point at the 17th monkey. **D–F**, Across all monkeys, the median PI is correlated ($p < 0.01$) with each of component listed in **B**: median set reached (**D**), average session duration (**E**), and average errors/trials (**F**).

using a series of two-choice discriminations. The ASST was used to assess individual differences in flexibility of action selection using sequences of visual discriminations of geometrical objects as previously published (Shnitko et al., 2017). Six weeks following MRI scans, all monkeys were simultaneously presented with photographs (fruit, conspecific animals, nature scenes, etc.) on their individual computer touch-screens within the home cages. Touching a photograph resulted in the picture enlarging to fill the full screen. The “training” sessions continued with 96 presentations of two side-by-side photographs per session. No food reinforcement was used to train animals to touch the image, and all animals reliably interacted with the touch-screens after five sessions (on average, $53 \pm 26\%$ of presentations resulted in touching one of the photographs during the last session, with fruit being the most consistently chosen or “preferred” category). Animals were then given 37 ASST sessions. During the first seven sessions, the monkeys learned to associate touching a geometric shape presented on the screen with the delivery of a 1 g banana-flavored pellet paired with presentation of a photograph of a fruit. A second order of food reinforcement after correct choice was introduced across the 7 d period. Initially, a fixed-ratio 1 (FR1) schedule was used in which a correct response resulted in presentation of a fruit photograph and a banana pellet. The FR for the pellet delivery was increased until FR3. After each pellet delivery, a small (1 cm³) banana icon appeared at the top of the screen as visual feedback for the count of correct trials in the second-order schedule. Following the 7 d “training” on ASST, the next 30 consecutive daily sessions were considered “test” sessions used to evaluate individual differences in ASST performance. As noted above, all monkeys in a cohort were simultaneously given ASST sessions and therefore had auditory and visual access to other monkeys performing the task.

ASST trials and sets. Schematic representation of a trial is depicted in Figure 1A. During the sessions, the monkeys initiated a trial by touching anywhere on the screen (displaying a nature photograph) and this response resulted in the presentation of two geometrical stimuli, side-by-side, right and left of center, comprising a “set.” Monkeys were allowed 30 s (limited hold) to respond on either stimulus. Each trial ended when the monkey touched one of the two stimuli or 30 s elapsed. The outcome of the trial depended on the monkey’s selection. A touch on the predetermined “correct” stimulus resulted in a 2 s presentation of a preferred photograph (e.g., an apple sliced in two), whereas an incorrect response resulted in a 10 s presentation of a black screen (i.e., a delay to next trial). Correct and incorrect stimuli were randomly altered between left or right sides. If a monkey did not touch either of the stimuli, a 10 s presentation of a black screen was imposed similar to an incorrect response. Criterion for acquiring the discrimination (i.e., learning the “set”) was a running total of 12 of 15 correct trials (as in Weed et al., 2008), and the odds of a correct trial was 50%. Once criterion was met, the next set was presented on the next trial. There was a total of 8 sets possible in a session (four original and four reversal). The sequence of discrimination sets remained consistent from session to session, although the shapes and colors were randomly chosen for each set that was not a reversal set (except the simple discrimination [SD] of Set 1 in which shapes were either both black or both white). Each session began with Set 1, regardless of the previous session performance, and the monkey could advance to Set 2 (Set 1-reversal [SD-R]) based on the above criterion. Set 3 introduced color as a variable (i.e., a compound stimulus) and was labeled as a compound discrimination with new shapes

and colors with the correct response based on shape, followed by Set 4 (Set 3-reversal). Set 5 was an intradimensional shift with new shapes and colors with the correct response based on shape, followed by Set 6 (Set 5-reversal). Set 7 was an extradimensional shift with new shapes and colors, but color dictated the correct response, followed by Set 8 (Set 7-reversal). The session ended on either the successful completion of the final set (which was always the reversal of Set 7) or 45 min elapsed from the start of session. No monkey was excluded from data collection based on the task performance.

Statistical analysis

Behavioral data. The subject-specific PI for each session was composed of three outcome measures or factors: (1) the ratio of the incorrect responses (errors) to trials per session, (2) the session duration (2700 s maximum), and (3) the final set reached by the end of session (Shnitko et al., 2017). Each of these three measures was scaled to 0–100 by calculating the minimum and maximum possible for each: session duration (192–2700 s), set reached (1–8), and ratio of session errors to session trials (0–1). The normalized measures were then summed to get an individual PI for a session (maximum of 300). The details of the scaling procedure have been previously described (Shnitko et al., 2017).

The main dependent variables of interest were as follows: the three variables comprising the PI (given above), the regression slope of PI over 30 consecutive daily sessions (representing subject-specific rates of task acquisition), trials to criterion (TTC) for completing a set, duration of each set, food pellets earned, proportion of correct responses during the first set, latency to touch a shape, (a measure of proactive response inhibition) (Meyer and Bucci, 2016), and the perseverative errors (the

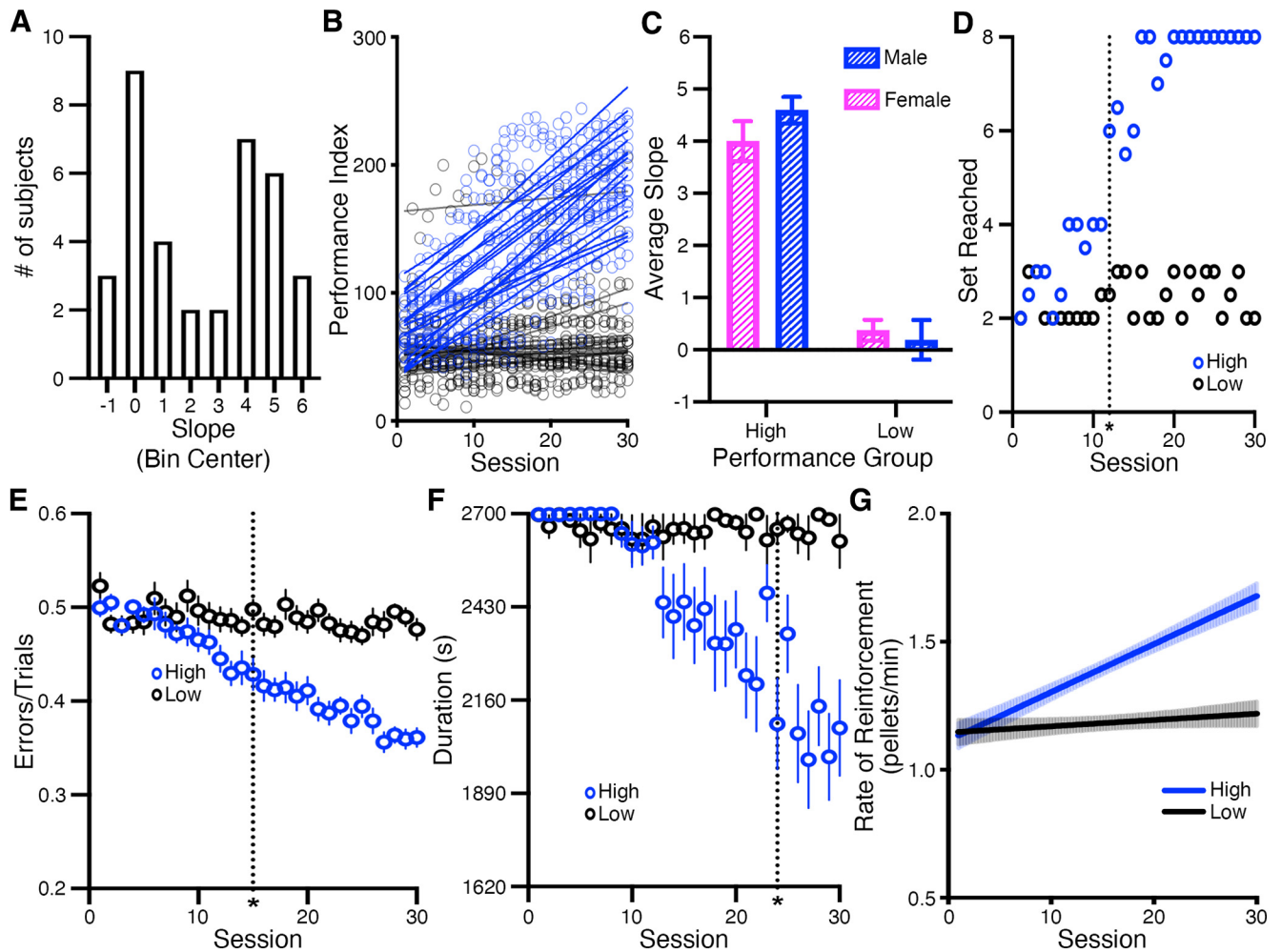


Figure 2. Classification of monkeys as high and low ASST performers based on the PI. **A**, Individual progression through the task was assessed using slopes of the regression between PI and consecutive sessions, showing a bimodal distribution of slopes. **B**, Change in PI on the ASST as a function of session for each monkey characterized as HPs (blue) and LPs (black). An outlier monkey (only monkey with a PI >150 on the first 5 sessions) showed High performance initially but did not substantially improve over time similar to Low performance and was therefore removed from analyses. **C**, Average regression slopes ($\pm$ SEM) of PI by sex for HPs ($n = 17$, 8 females) and LPs ($n = 17$, 10 females). **D**, High and Low group median set reached across the 30 sessions. **E**, High and Low group average errors per trial ($\pm$ SEM) in each session. **F**, High and Low group average session duration ($\pm$ SEM) in each session. Vertical dotted lines at Sessions 12, 15, and 24 (**D–F**, respectively) indicate when the two groups first show a significant difference and remain different over the remaining sessions. * $p < 0.05$ (adjusted t test). **G**, Average rate of pellet delivery across 30 sessions for HPs and LPs. Shadow areas represent 95% CI.

number of consecutive incorrect trials after the first incorrect trial) (Sandson and Albert, 1984, 1987; Shnitko et al., 2019).

A k -means cluster analysis with the number of clusters equal to 2 was used to classify subjects based on the slope of the PI over the 30 sessions. The decision to use 2 clusters (i.e., 2 groups: HPs and LPs) in the data analyses was based on the bimodal distribution of the individual slopes (see Fig. 2A). When >2 clusters were used in the k -means cluster analysis, the number of subjects were distributed as follows: three clusters: $n = 9, 16, 10$; four clusters: $n = 7, 8, 4, 16$. These two clusters differed on many dimensions of the ASST (see Figs. 2, 3) as well as variables not included in the PI, such as rate of reinforcement, response latency, and perseverative errors.

ASST and dual-regression analysis of RSNs. The four signal-ICs (RSNs) were subjected to a dual-regression analysis to identify group-dependent differences in the resting-state functional connectivity between the LP and HP groups (Beckmann et al., 2009; Veer et al., 2010) within FSL. Voxel-wise z scores of connectivity with a RSN (dual-regression Stage 2 maps, $p < 0.05$ threshold) (Nickerson et al., 2017) were extracted for each subject and averaged across voxels within an ROI. This measure of ROI connectivity with a RSN was used as a dependent variable in a two-way ANOVA with performance group and brain hemisphere as factors. Specifically, multiple linear regression of z scores in group-ICA 4D maps (the output of MELODIC processing) against the

individual 4D datasets produced variance normalized time courses for each of the four components and subject (des norm = 1 was specified in the dual-regression command). Voxel-wise statistical differences between experimental groups were estimated using the FSL randomize tool with threshold-free cluster enhancement and 500 random permutations (Nichols and Holmes, 2002; Smith and Nichols, 2009). In order to identify group differences within each of the four signal-ICs, the resulting statistical maps were set at a threshold of $p < 0.05$ (TFCE-corrected for family-wise errors). The statistical maps with significant differences between groups were used to extract individual subject z scores from subject-specific spatial maps for subsequent statistical analysis of individual differences in the ROI-to-RSN connectivity and cognitive measures. The ROI voxel-average connectivity was used in a correlative analysis to identify a relationship between ROI connectivity with an RSN and behavioral measures of proactive response inhibition and continuous perseveration. Comparison between HPs and LPs was performed using t tests corrected for multiple comparison with Bonferroni adjustment.

One subject was an outlier in ASST because of high performance on the first session with no further improvement (described in detail in Results and shown in Fig. 2B). Thus, the statistical analysis of the behavioral measures and resting-state data was performed with ($n = 35$) and without ($n = 34$) this subject. No significant differences in behavioral and

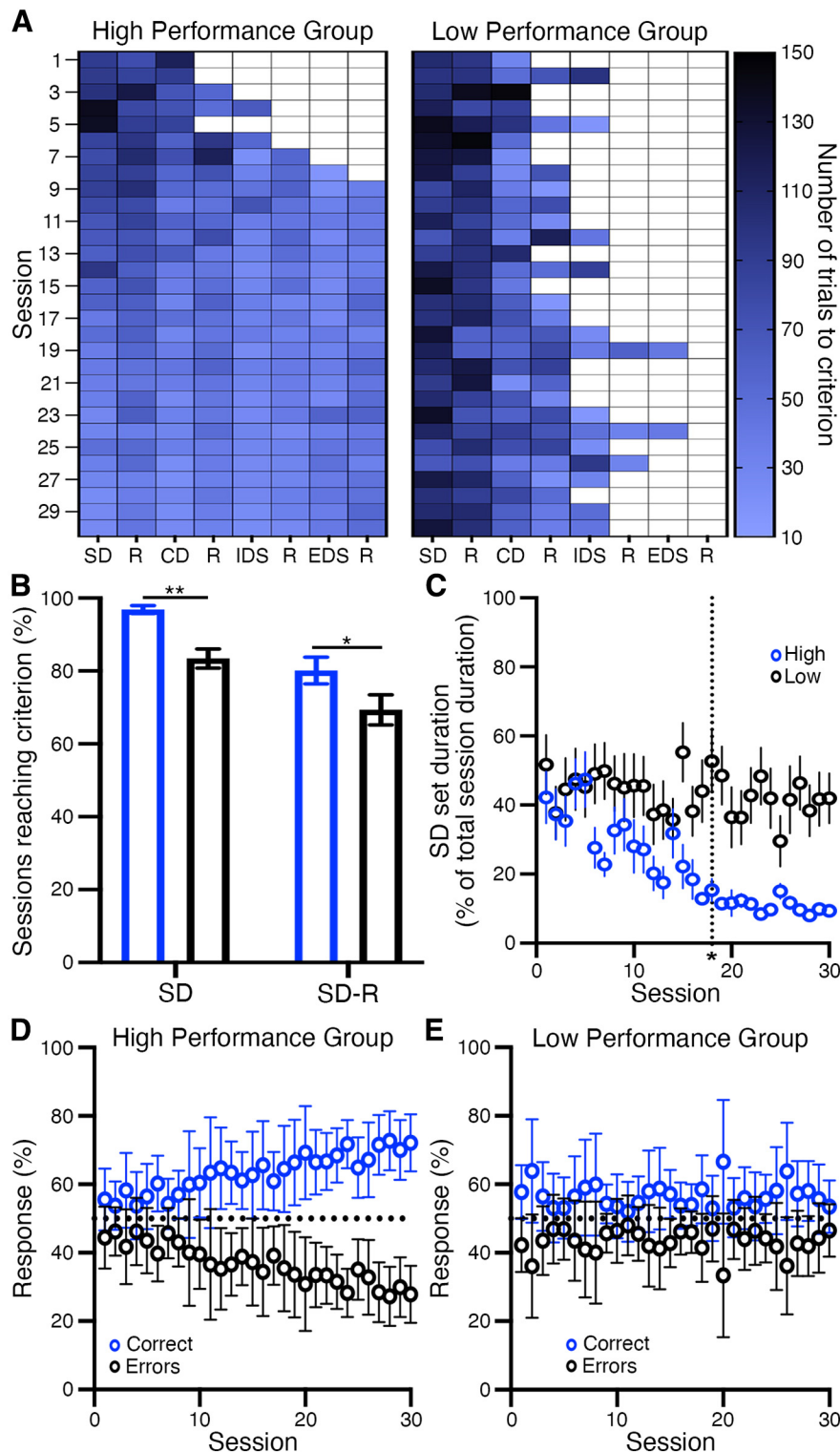


Figure 3. Group differences in improvement on the three measures in the PI. **A**, Average TTC plotted by session (y axis) and set (x axis) for high (left graph) and low (right graph) performers. Each cell within a matrix is the average trials for all individuals in each group that met the criterion for the corresponding set. Empty cells indicate no monkey within the group reached the criterion for that set during that session. **B**, Average ($\pm$ SEM) percentage of sessions to reach criterion for SD and SD-R for High (blue) and Low (black) performers. $**p < 0.01$ (Sets); $*p < 0.05$ (Group); t test of groups. **C**, Percentage of SD set duration of the total session duration across 30 sessions for High (blue) and Low (black) performers. Dotted vertical line and asterisk (at Session 18) indicate consistent group differences for remaining sessions. **D**, **E**, Group average ($\pm$ SEM) of the percentage of correct and erroneous responses during the SD (Set 1) in each of the 30 sessions for high (**D**) and low (**E**) performers. Dotted horizontal line in **D** and **E** indicates 50% of responses.

imaging results were observed between these two approaches. The results of analysis performed without the outlier are reported.

Results

Individual differences rhesus monkeys based on ASST performance

Three measures were collected during ASST for each monkey in each session: the ratio of errors to trials, the final set that a monkey reached by the end of a session, and the session duration (Fig. 1B). These measures were used as factors to calculate a composite PI score calculated for each monkey and session, as previously described (Shnitko et al., 2017). The 35 monkeys were then rank-ordered with higher PIs corresponding to higher performance (Fig. 1C). Figure 1D–F shows correlations between each factor and a median PI for subjects across 30 sessions (all $R^2 > 0.87$, all $p < 0.0001$, simple regression analysis). Monkeys with higher PIs during ASST tended to reach all 8 sets, reaching a higher median set (Fig. 1D) within a short period of time (Fig. 1E) and with a small number of errors during the sessions (Fig. 1F). Importantly, a single-factor analysis was insufficient to assign individual ranks to each of the 35 because of overlapping values.

Classifying monkeys based on the rate of ASST acquisition

Individual improvement in PI on the ASST across the daily sessions was fitted to a regression line to represent an individual rate of task acquisition. Figure 2A shows a bimodal distribution of individual slopes that suggested two groups of subjects. The slope of each regression line (or rate of PI improvement) was used to classify monkeys as HP and LP using a k -means cluster analysis (see Materials and Methods). Two distinctive patterns in the acquisition rates were obtained and are highlighted by blue (High, $n = 17$) and black (Low, $n = 17$) colored plots (Fig. 2B). One subject who was classified as an LP was an outlier because of the high performance on the first session, without improvement over the 30 sessions (Fig. 2B, top-most plot in black). The group average rate of improvement was 4.3 ± 0.9 PI/session in the HPs and 0.3 ± 0.8 PI/session in the LPs. There was no significant difference in the rate of the ASST acquisition between male and female monkeys (Fig. 2C, two-way ANOVA: group (high and low), $F_{(1,30)} = 178.2$, $p < 0.0001$; sex, $F_{(1,30)} = 0.45$, $p = 0.5$; group $\times$ sex, $F_{(1,30)} = 1.7$, $p = 0.2$).

The group-dependent differences in the ASST acquisition between the HPs and LPs were characterized by significant group differences in the three factors composing the PI. Figure 2D illustrates the highest set reached for 50% of the LPs (i.e., the median) was the third set that followed the first reversal set and added compound stimuli of shapes and colors. In contrast, the HPs steadily increased in the set reached across the first 20 sessions with all 8 sets routinely reached thereafter. The group difference in set reached (High vs Low) was significantly different by Session 12 (Fig. 2D; two-way repeated-measures ANOVA: group, $F_{(1,33)} = 46.1$, $p < 0.0001$; session, $F_{(10,356)} = 20.1$, $p < 0.0001$; group $\times$ session, $F_{(29,986)} = 14.9$, $p < 0.0001$; t test: High > Low, all $t > 19.4$, all adjusted $p < 0.05$ from Session 12 to Session 30, with the exception of Session 14 with $p = 0.79$). The LPs had consistently high levels of errors/trials over 30 sessions (Fig. 2E; range from 0.52 ± 0.06 to 0.47 ± 0.05). Compared with the LPs, the HPs decreased the ratio of errors per trial from 0.49 ± 0.04 (Session 1) to 0.36 ± 0.04 (Session 30; two-way repeated-measures ANOVA: group, $F_{(1,33)} = 25.2$, $p < 0.0001$; session, $F_{(13,437)} = 15.9$, $p < 0.0001$; group $\times$ session, $F_{(29,986)} = 10.8$, $p < 0.0001$; t test: High > Low, all $p < 0.05$ from Session 15 to Session 30, with the exception of Session 16 with $p = 0.073$; Fig. 2E). Finally, there were significant differences in the session duration between the two groups of monkeys. The time needed to complete the task decreased in the High performing monkeys from 2700 ± 3.4 s (Session 1) to 2080 ± 591 s (Session 30; Fig. 2F). The average session duration of the LPs did not change across the 30 sessions (2700 ± 3.6 s) and was significantly longer than the average session duration of the HPs (two-way repeated-measures ANOVA: group, $F_{(1,33)} = 12.2$, $p < 0.01$; session, $F_{(7,251)} = 8.3$, $p < 0.0001$; group $\times$ session, $F_{(29,986)} = 8.2$, $p < 0.0001$; t test: High > Low, all $p < 0.05$ from Session 24, with the exception of Session 25 with $p = 0.36$). Both groups gained a similar average number of pellets each session (Low = 53 ± 3 pellets and High = 55 ± 3 pellets), and this was consistent across 30 sessions ($R^2 < 0.005$, $p = 0.1$; data not shown). However, the HPs increased the rate of food reinforcement from 1.1 to 1.6 pellets/s ($R^2 = 0.24$, $p < 0.001$) over the 30 sessions while the rate of reinforcement in the LPs remained stable ($R^2 = 0.005$, $p = 0.1$). The difference in the change of rate of reinforcement across 30 sessions was significant between the groups ($F_{(1,33)} = 61.3$, $p < 0.0001$; Fig. 2G).

An additional analysis of errors showed that the LPs had a greater proportion of perseverative errors (0.24 ± 0.01 perseverative/total errors) compared with the High group (0.21 ± 0.02 perseverative/total errors, Mann–Whitney $U = 42$, $p < 0.001$, two-tailed; see Fig. 7A). An analysis of the latency to respond (action selection) showed that the median response latency was significantly faster in the Low (0.7 ± 0.3 s) compared with the HPs (1.3 ± 0.5 s, Mann–Whitney $U = 48$, $p < 0.001$, two-tailed; see Fig. 8A).

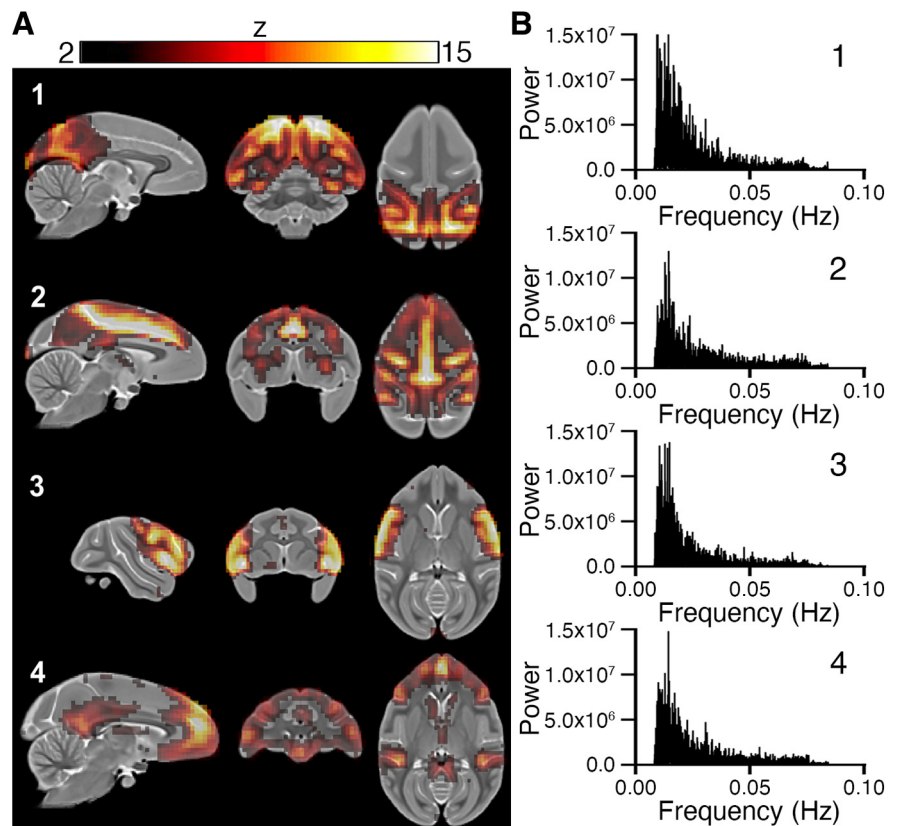


Figure 4. ICS identified under isoflurane anesthesia. **A**, T₂-weighted image of rhesus macaques' brain template (Weiss et al., 2021) with overlying ICS. The ICA was performed on AFNI processed data (Extended Data Fig. 4-1). The spatial maps are displayed at a threshold of $Z > 2$ for visualization purposes. These ICS are as follows: 1, Visual Network; 2, Motor Network; 3, Sensory-Motor Network; 4, ECN. **B**, Power spectral density of the 4 signal-ICS.

HP and LP differences in discriminative and reversal learning in the ASST

Overall performance differences between the HPs and LPs in the 30 ASST test sessions are shown in Figure 3A as a modified heat map of the average number of trials to reach criterion/session across all 8 sets (x axis) and across all 30 sessions (y axis). Each cell within the matrices represents the average number of TTC for those monkeys that were able to reach the criterion for a set. At the beginning of the ASST acquisition (Sessions 1–5), both groups required a similarly large number of TTC for the initial sets within a session (Set 1 to Set 3). After Session 6, the High performing group had lower average TTCs in each set and by Session 9 consistently reached the eighth set within a session.

To address common measures of learning and flexibility in ASST (i.e., acquiring a discrimination and its reversal), comparisons between groups for all 30 sessions is possible only for the first two sets. The HPs had a greater proportion of sessions that met criterion for the SD (Set 1–SD) and its reversal (Set 2–SDR), $96 \pm 4\%$ and $79 \pm 14\%$, respectively, compared with the LPs that reached criterion for Set 1 and Set 2, $83 \pm 11\%$ and $69 \pm 17\%$ of the 30 sessions, respectively (Fig. 3B; two-way ANOVA: group, $F_{(1,33)} = 13.5$, $p < 0.001$; Set, $F_{(1,33)} = 28.3$, $p < 0.0001$; group $\times$ set, $F_{(1,33)} = 0.21$, $p = 0.65$). The group-dependent differences in the number of sessions to criteria were reflected in the proportion of errors that were perseverative errors in the sessions where criterion for Set 1 and Set 2 was reached. Specifically, the average proportion of perseverative errors across the 30 sessions in the HPs was 0.12 ± 0.06 for Set 1 and 0.23 ± 0.04 for Set 2, compared with the 0.2 ± 0.06 for Set 1 and 0.26 ± 0.05 for Set 2 for

the LPs (two-way repeated-measures ANOVA: group, $F_{(1,33)} = 18.5$, $p < 0.001$; Set, $F_{(1,33)} = 46.4$, $p < 0.0001$; group $\times$ set, $F_{(1,33)} = 3.0$, $p = 0.09$, data not graphed).

In terms of time efficiency, the LPs steadily spent $\sim 43\%$ of the session time (range 30%–55%) acquiring Set 1-SD across the 30 sessions (Fig. 3C). In contrast, the HPs decreased the time spent to reach criterion for Set 1-SD from 44% (Session 1) to 15% (Fig. 3C). There was a main effect of group (High vs Low, two-way repeated-measures ANOVA: group, $F_{(1,34)} = 33.8$, $p < 0.001$), session ($F_{(13,442)} = 2.6$, $p < 0.01$), and group $\times$ session interaction ($F_{(29,986)} = 1.7$, $p < 0.01$). *Post hoc* analysis of Set 1-SD duration found HPs had shorter latencies compared with LPs in Session 25 to Session 30 (High < Low, all $t > 3.7$, all adjusted $p < 0.05$; Fig. 3C). HPs were also more efficient as measured by the proportion of correct trials on the last session ($72 \pm 8\%$ on Session 30; Fig. 3D). In contrast, LPs remained at near chance levels with an average proportion of correct responses of $58 \pm 8\%$ on Session 1 $54 \pm 8\%$ on Session 30 (Fig. 3E).

Resting-state networks identified in anesthetized rhesus monkeys

ICA identified 10 components, and Figure 4A shows, coronal and axial slices of a monkey brain with spatial maps of the four signal-ICs (or networks) identified by MELODIC. The remaining ICs (5–10) were classified as noise-ICs and are shown in Figure 5. The classification of the ICs as signal versus noise networks was performed based on the visual inspection of the spatial maps (Figs. 4A, 5A) and analysis of the time course power spectra (Figs. 4B, 5B). These ICA maps show a low number of large clusters of the brain regions greatly overlapping with neural (gray matter) areas. The distribution of power shows predominantly low frequency with peaks between 0.01 and 0.025 Hz for each of IC 1–4. The spatial maps were used to identify the relative contribution of 55 brain regions (Weiss et al., 2021) within each of the four networks. The ROIs within each network were as follows: IC 1, Visual Network, centered on the occipital cortex (V1, V2, V3, V3a, and V4), parietal and posterior cingulate cortex; IC 2, Motor Network, included the supplementary (F6, F3), primary (F1) and dorsal premotor (F2, F7) areas and putamen; IC 3, Sensory-Motor Network, incorporated ventral premotor (F4, F5), somatosensory (BA 1–2, 3a/3b) and substantial part of the dorsolateral prefrontal (BA 46, 9d, 8d,) cortices and putamen; and IC 4, Executive Control Network (ECN), centered on the medial and lateral prefrontal cortices as well as orbitofrontal cortex (OFC) and caudate nucleus.

Neurocircuitry underlying differences in HPs and LPs identified with dual-regression analysis

Dual-regression analysis of the four signal-ICs revealed stronger functional connectivity within the ECN in HPs compared with LPs (Fig. 6). There were no group-dependent differences in the functional connectivity with the remaining 3 (visual, motor, and sensory-motor) networks. The spatial maps in Figure 6A–C show voxels in red that exhibited greater levels of correlation between fluctuation of their signal over time (time-series) with the average (across all voxels within the network) time-series of ECN (nominally the functional connectivity) in the HPs compared with the LPs. In the striatum, the anterior caudate had a robust number of voxels ($n = 27$ in the left and $n = 4$ in the right hemisphere) with a greater level of correlation with the ECN in the HPs compared with the LPs (Fig. 6D). In the HPs, the average z score for caudate voxels with significantly greater functional connectivity to the ECN was 2.2 ± 0.6 (left) and $2.7 \pm$

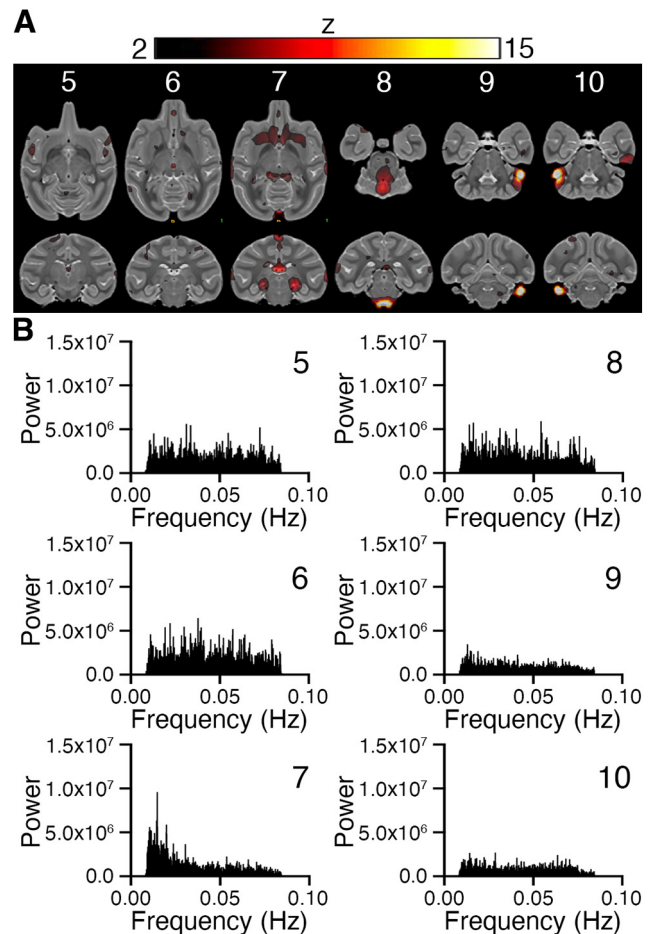


Figure 5. ICs 5–10 identified as noise. ICs were identified as noise based on the criteria in Duff et al. (2018). **A**, T_2 -weighted image of rhesus macaques' brain template (Weiss et al., 2021) with overlying IC 5–10 spatial maps. The spatial maps are displayed at a threshold of $Z > 2$. **B**, The graphs of power spectral density for each component show almost even distribution of signal across represented frequencies without strong peaks, with the exception of IC 7. However, analysis of the spatial map for this component revealed large overlap with ventricles and the sagittal sinus.

0.5 (right). In contrast, the level of synchronization with the executive control network was significantly lower in the LPs [$z = 0.2 \pm 0.4$ (left) and $z = 0.3 \pm 0.3$ (right); two-way ANOVA: group, $F_{(1,64)} = 19.9$, $p < 0.0001$; hemisphere, $F_{(1,64)} = 0.5$, $p = 0.5$; group $\times$ hemisphere, $F_{(1,64)} = 0.1$, $p = 0.7$] (Fig. 6D). Similar to the caudate, the putamen's resting state activity in the LPs ($n = 4$ voxels left hemisphere and $n = 6$ voxels right hemisphere) was also asynchronized with the ECN [$z = -1 \pm 0.4$ (left) and $z = -0.5 \pm 0.4$ (right)], which was different from that of the HPs [$z = 1 \pm 0.4$ (left) and $z = 1.6 \pm 0.6$ (right); two-way ANOVA: group, $F_{(1,64)} = 17.8$, $p < 0.0001$; hemisphere, $F_{(1,64)} = 1.3$, $p = 0.26$; group $\times$ hemisphere, $F_{(1,64)} = 0.01$, $p = 0.9$] (Fig. 6E).

In the prefrontal cortical areas, the OFC and ventrolateral PFC (vLPFC) were correlated with the ECN in both the HPs and LPs; however, group-dependent differences were present (Fig. 6F,G). Average z scores for the OFC (voxels' $n = 3$ in the left and $n = 4$ in the right hemispheres) were 3.2 ± 0.3 (left) and 2.9 ± 0.6 (right) in the HPs and 1.4 ± 0.3 (left) and 1.1 ± 0.4 (right) in the LPs. The difference between groups was significantly different (two-way ANOVA: group, $F_{(1,64)} = 18.4$, $p < 0.0001$; hemisphere, $F_{(1,64)} = 0.5$, $p = 0.5$; group $\times$ hemisphere, $F_{(1,64)} = 0.003$, $p = 0.9$) (Fig. 6F). Similar results were observed with the vLPFC (voxels' $n = 6$ in the left and $n = 7$ in the right hemispheres),

where average z scores were 4.6 ± 0.7 (left) and 5.5 ± 0.6 (right) in the HPs and 1.5 ± 0.4 (left) and 2.5 ± 0.4 (right) in the Low group (two-way ANOVA: group, $F_{(1,64)} = 30.3$, $p < 0.0001$; hemisphere, $F_{(1,64)} = 2.6$, $p = 0.1$; group $\times$ hemisphere, $F_{(1,64)} = 0.005$, $p = 0.9$) (Fig. 6G). Group-dependent differences were also identified in functional connectivity to the ECN with the right hemisphere for the ventromedial PFC (vmPFC; voxels $n = 7$; Fig. 6H) and the superior temporal cortex (STC; voxels $n = 2$; Fig. 6I), with higher average z scores for both brain areas in the HPs (unpaired t test, all $t > 2.8$, $df = 32$, all $p < 0.01$).

Two aspects of behavior, perseveration and impulsivity, which are frequently associated with automatized behavior, were investigated for associations with specific areas of connectivity in the ECN. Perseverative responding was associated with the strength of connectivity for 4 of 6 brain regions showing differential correlation with the ECN between the two groups (Fig. 7). Specifically, greater functional connectivity of caudate, putamen, vlPFC, and STC (Fig. 7B–E, respectively) with the ECN network correlated with a lower proportion of perseverative errors (all $R^2 > 0.18$, all $F_{(1,32)} > 7$, all $p < 0.05$). Latency to respond, a measure of proactive response inhibition, scaled with connectivity for 5 of the 6 brain regions showing differential functional connectivity to the ECN between two groups (Fig. 8A). Specifically, longer latencies (i.e., greater inhibition) was associated with greater functional connectivity of the caudate, putamen, vlPFC, vmPFC, and OFC (Fig. 8B–F, respectively) with the ECN (all $R^2 > 0.11$, all $F_{(1,32)} > 4.3$, all $p < 0.05$).

Discussion

The novel ASST procedure identified two distinct approaches by colony-bred rhesus monkeys toward performing the task. One approach was a predisposition toward autonomous habitual behavior that randomly resulted in reinforcement (LP monkeys), and the other approach was a predisposition toward acquiring a new response when the basis of the stimulus association with reinforcement changed (HP monkeys). Overall, the HP monkeys were able to flexibly adjust action selection when contingencies change, and to become more efficient in advancing through the sets compared with the LP monkeys (Fig. 2). Although both HP and LP monkeys obtained similar amounts of food during each session, these populations differed in several outcome measures, including the PI (reflective of errors/trial, set reached, and time to finish 8 sets), the rate of food reinforcement, the number of perseverative errors, and the latency to respond.

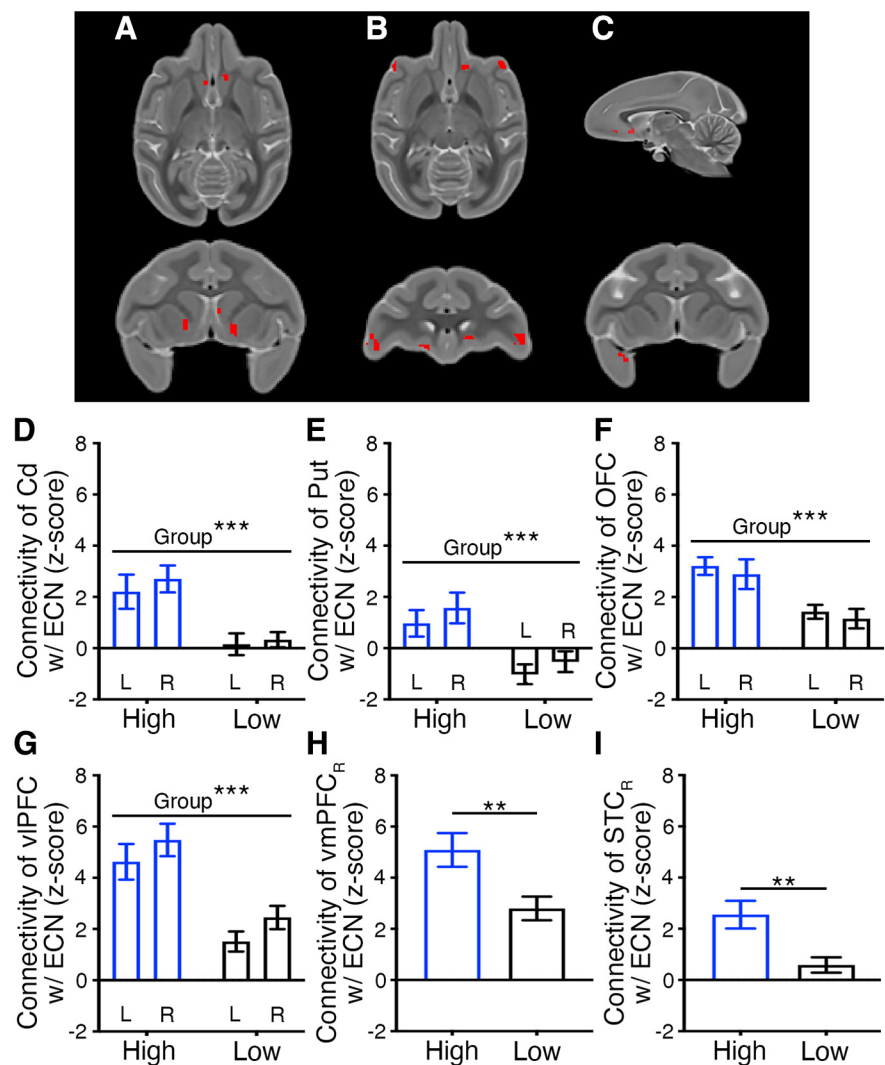


Figure 6. Neurocircuitry associated with high versus low performance in rhesus monkeys. **A–C**, T₂-weighted image of the ONPRC 18 template (Weiss et al., 2021) with labeled voxels (red) in the striatum (**A**), the OFC and vlPFC (**B**), and vmPFC and STC (**C**) in which functional connectivity with IC 4 was greater in the HP group compared with the LP group (family-wise error corrected $p < 0.05$). **D–I**, Average (mean $\pm$ SEM) strength of connectivity of the caudate (Cd, **D**), putamen (Put, **E**), OFC (**F**), vlPFC (**G**), vmPFC of the right hemisphere (vmPFC_R, **H**), and STC of the right hemisphere (STC_R, **I**) with the ECN in the left (L) and right (R) hemispheres for high (blue) and low (black) performers. Stage 3 dual-regression group-average map for IC 4 (ECN) with corrected p values (High > Low) was used to extract Z scores for voxels with $p < 0.05$ for each individual subject. *** $p < 0.001$; ** $p < 0.01$; main effect of group (two-way ANOVA).

Compared with LP monkeys, the HP monkeys progressed through the 8 sets faster and with less errors per trial (Fig. 2E,F). Specifically, HP monkeys on average acquired the first two discrimination sets (SD and its reversal) in $\sim 90\%$ and 80% of the 30 test sessions, respectively, thereby excelling in conventional measures of cognitive flexibility (Clarke et al., 2008). In contrast, the LP monkeys met criteria for the first set in 80% of the 30 sessions (Fig. 3B), but the percentage of correct trials was only slightly above chance (i.e., 50%) in each session (Fig. 3E), and they spent $\sim 50\%$ of the session time in this set (Fig. 3C). Although the LP monkeys were inefficient during the first set, they successfully reached the second set (Set 1 reversal [SD-R]) in $\sim 70\%$ of the sessions and rarely advanced further. Between group comparisons of response errors in the first two sets found the LP monkeys made more perseverative responses, a traditional measure of behavioral inflexibility across species (Head et al., 2009; Woicik et al., 2011; Brown

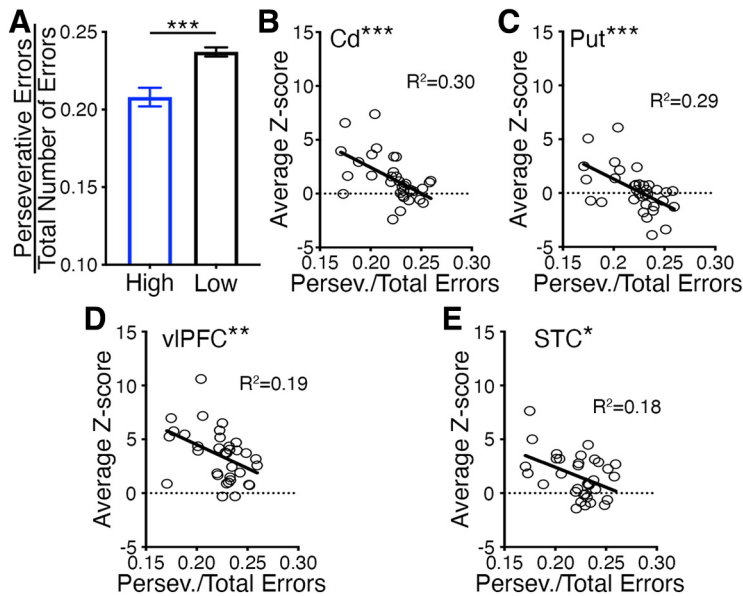


Figure 7. Brain region associations with perseverative errors during ASST performance. **A**, Average (mean $\pm$ SEM) proportion of perseverative errors to total errors across all sets and sessions for high (blue) and low (black) performers. *** $p < 0.001$ (unpaired t test). **B–E**, Linear regression of the proportion of perseverative errors and the strength of connectivity of the ECN with the caudate (Cd, **B**), putamen (Put, **C**), vIPFC (**D**), and STC (**E**). * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

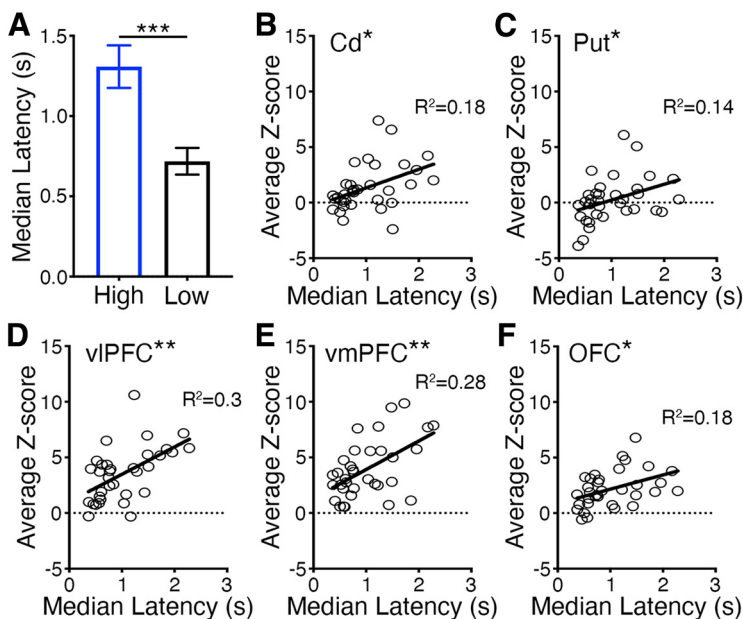


Figure 8. Brain region associations of response latency during ASST performance. **A**, Group differences in latency to touch a shape (average of median latencies $\pm$ SEM). *** $p < 0.001$ (unpaired t test). **B–F**, Correlations of each monkey's functional connectivity of caudate (Cd, **B**), putamen (Put, **C**), vIPFC (**D**), vmPFC (**E**), and OFC (**F**) with the ECN (average Z score) and their response latency. * $p < 0.05$. ** $p < 0.01$.

and Tait, 2016). Another contributing factor in LP performance was faster latencies to touch a stimulus, which is a common measure of rapid-response impulsivity in humans (Jauregi et al., 2018) and an example of a deficit in proactive response inhibition (Meyer and Bucci, 2016). Together, LP monkeys did not form a strategy to guide the discrimination and enable generalizations to new associations, a deficit similar to the performance of obsessive-compulsive individuals in ASST (van Beilen et al., 2005; Vaghi et al., 2017).

The bimodal distribution of performance that identified two population distributions of monkeys was likely because of a number of the experimental parameters. Unlike previous ASST procedures using Cambridge Automated Neuropsychological Test Automated Battery with monkeys or humans (Taffe et al., 2004; Wright et al., 2013; Wright and Taffe, 2014; Vaghi et al., 2017), the main dependent variable was the slope of PI over the 30 sessions, capturing the learning phase of performing the ASST (i.e., not training to a set criteria). In addition, the sessions always began at the first set, which enable a direct comparison of behavior in at least one set across all animals. The current ASST also provided the opportunity for obtaining food that was not dependent on sequential correct responses, as correct trials were reinforced under a second-order schedule of reinforcement that kept the LP monkeys engaged in the task for the duration of the session independent of acquiring the discrimination. The task parameters also allowed the HP monkeys that successfully learned the task (i.e., appeared model-based) to advance quickly through the sets and increase their rate of reinforcement by avoiding the 10 s timeout following an incorrect response. A delay of reinforcement is known as a particularly potent factor for influencing an individual's choice (Mazur, 1997), and the HP monkeys steadily increased the rate of pellet delivery over the 30 sessions (Fig. 2G). However, the 10 s timeout following an incorrect response may also introduce a delayed memory component to this ASST that was experienced by the LP animals in nearly half of their trials. In future studies, removing the timeout will be an informative manipulation to address the role of memory versus sensitivity to negative reinforcement (active avoidance of the delay) in ASST performance. Finally, the influence of the social setting may have been a factor in performing the task. Testing members of a cohort simultaneously within the home cage in the housing room provided an array of ongoing activity that could compete for attention to the task. For example, an easily distracted monkey may choose to attend to other monkeys engaging in the ASST and respond randomly or with a side-preference when the stimuli are presented. In summary, the ASST performance may be influenced by sensitivity to the rate of food reinforcement, longer intertrial intervals, or attention deficits. The bimodal distribution in the slope of PI increase over sessions suggests that these sensitivities are reflective of underlying trait-like characteristics in advancing through the ASST (Shnitko et al., 2019; Grant et al., 2021).

To address functional differences in neural circuits underlying the HP or LP classification, large-scale RSN analysis was conducted with a data-driven ICA approach, which is widely used to assess brain neurocircuitry in both humans (Beckmann et al.,

2005; Smith et al., 2009; Allen et al., 2014; Finn et al., 2015) and laboratory animals (Hutchison et al., 2011, 2014; Yacoub et al., 2020). The four RSNs identified here include a diverse set of brain regions (Fig. 4), drawn from a large number of subjects, an extensive (30 min) time-series for fMRI acquisition, and the low motion artifact with 1% isoflurane anesthesia (Hutchison et al., 2014). Each of the networks in the present study is a combination of two or three RSNs that have been described in human studies. For example, the visual network identified in this study incorporated medial, occipital pole, and lateral visual areas (or Maps 1, 2, and 3) observed in the Smith et al. (2009) study. This might be because of the impact of isoflurane anesthesia on resting-state connectivity. In humans, anesthetic agents, such as isoflurane, can result in both increased or decreased functional connectivity, and the impact depends on the RSN (Martuzzi et al., 2010; Lv et al., 2016). In macaque monkeys, increasing isoflurane from 1.0% to 2.75% decreases the overall correlation magnitude both globally and locally but does not change the underlying network structure (Areshenkoff et al., 2021). Likewise, in marmosets, 1.5% isoflurane anesthesia decreased the strength of connectivity within RSNs; however, the brain regions identified in the networks were preserved (Hori et al., 2020). Thus, while general anesthesia decreases levels of synchronized activation between brain regions, it allows for MRI data acquisition conditions across a large number of monkeys that can be used to address circuitry underlying populational traits guiding correlated behavior.

Greater functional connectivity of cortical or striatal components with the ECN of HP animals provides perspective on the neurocircuitry underlying increased behavioral flexibility of this group compared with LP monkeys. The ECN identified with ICA on these monkeys contains brain regions within three major RSNs associated with cognitive function in humans (executive control, frontal-parietal, and default mode networks) (Vaghi et al., 2017; Zhao et al., 2019). In humans, the ECN incorporates the middle prefrontal, anterior cingulate, and ventrolateral prefrontal cortices (Beckmann et al., 2005). These brain regions correspond to major cognitive domains underlying executive control (Miyake et al., 2000) and are active during tasks assessing working memory (Curtis and D'Esposito, 2003), response inhibition (Aron, 2007), and set shifting (Dreher and Berman, 2002). In agreement with this evidence, monkeys classified as HP on the ASST showed enhanced functional connectivity of the vIPFC and vmPFC with the ECN. This result indicates that greater functional connectivity between these cortical areas might underlie enhanced associative functioning in the HP monkeys that aided in their consistent improvement over sessions. Additionally, the HP animals had greater functional connectivity of the caudate, putamen, and OFC with the ECN. While these brain regions have not been reported to be a part of a distinct RSN (Smith et al., 2009; but see Robinson et al., 2009), they have been repeatedly linked to the control over associative and reversal learning, cognitive flexibility, and response inhibition (Kim et al., 2012; Antzoulatos and Miller, 2014; Zhang et al., 2017; Hung et al., 2018).

A prevailing hypothesis is that cortical synchrony with the dorsal striatum (i.e., caudate and/or putamen) reflects cognitive flexibility (Seo et al., 2012; Vaghi et al., 2017). Human fMRI studies have identified the vIPFC/OFC connectivity with the caudate as a critical element in goal-directed planning (Monchi et al., 2007; Graham et al., 2009). Additionally, cognitive flexibility has been shown to be encoded in neurons of the medial striatum (Kimchi and Laubach, 2009) and suggested to be dependent on

connections between the OFC and medial striatum (Clarke et al., 2008). Humans with obsessive compulsive disorder display reduced cognitive flexibility assessed with ASST that was associated with reduced functional connectivity between the caudate and the vIPFC and increased local connectivity of the putamen compared with healthy controls (Vaghi et al., 2017). In agreement with this finding, both the caudate and vIPFC showed high bilateral connectivity with the ECN in HP monkeys (Fig. 6D,G), a finding reflected in the negative correlation of ECN connectivity of vIPFC and caudate with perseverative errors (Fig. 7B,D). The putamen, on the other hand, is traditionally recognized as a mediator of automatic habitual behavior (Botvinick et al., 2009; Redgrave et al., 2010; Dezfouli and Balleine, 2013; Kim and Hikosaka, 2015). Indeed, the putamen showed diminished connectivity with the ECN in the LP monkeys (Fig. 6E). However, the present data also show significant connectivity of the rostral putamen with the ECN in the HP monkeys (Fig. 6E). This novel finding of cortical-putamen positive connectivity associated with improved ASST performance agrees with anatomic studies in both humans and NHPs showing that rostral part of the putamen receives input from the prefrontal, presupplementary motor, and temporal cortices (Yeterian and Pandya, 1998; Middleton and Strick, 2000; Lehericy et al., 2004; Kim and Hikosaka, 2015).

In conclusion, this ASST is an efficient, informative, and robust method to attain a reliable and valid measure of behavioral flexibility (Shnitko et al., 2017, 2019; Grant et al., 2021). By characterizing a large number of colony-bred rhesus monkeys on the ASST, two populations (HP and LP) were identified that differed fundamentally on their performance on the ASST as well as in fMRI RSNs found by ICA. Differences between HP and LP monkeys primarily reside in brain connectivity within the ECN, and their characterization allows for pinpointing brain regions that were previously linked to cognitive flexibility and response inhibition in human fMRI. Particularly informative was the strong connectivity between the dorsal striatal subregions with the ECN that was observed in individuals with reduced response perseveration and higher response inhibition reflecting greater flexibility in task performance. Importantly, performance on this ASST also robustly predicts future heavy alcohol drinking in the rhesus monkeys (Shnitko et al., 2019; Grant et al., 2021). Because both ASST and MRI can support longitudinal designs, the combination can be useful in identifying trait-like risks for and associated brain circuitry for developing prevention or therapeutic interventions.

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