Regional, Layer, and Cell-Type-Specific Connectivity of the Mouse Default Mode Network

Graphical Abstract

Highlights

- Mouse resting-state default mode network anatomy described at high resolution in 3D
- Systematic axon tracing shows cortical DMN regions are preferentially interconnected
- Layer 2/3 DMN neurons project mostly in the DMN; layer 5 neurons project in and out
- Retrosplenial cortex contains distinct types of in- and out-DMN projection neurons

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In Brief

The default mode network is vulnerable to brain disorders, but details of its anatomy and connectivity are coarse. Whitesell et al. use modern neuroanatomical tools in the mouse, including whole-brain imaging and viral tracing, to provide high-resolution anatomical descriptions and identify cell type correlates of this conserved brain network.
SUMMARY

The evolutionarily conserved default mode network (DMN) is a distributed set of brain regions coactivated during resting states that is vulnerable to brain disorders. How disease affects the DMN is unknown, but detailed anatomical descriptions could provide clues. Mice offer an opportunity to investigate structural connectivity of the DMN across spatial scales with cell-type resolution. We co-registered maps from functional magnetic resonance imaging and axonal tracing experiments into the 3D Allen mouse brain reference atlas. We find that the mouse DMN consists of preferentially interconnected cortical regions. As a population, DMN layer 2/3 (L2/3) neurons project almost exclusively to other DMN regions, whereas L5 neurons project in and out of the DMN. In the retrosplenial cortex, a core DMN region, we identify two L5 projection types differentiated by in- or out-DMN targets, laminar position, and gene expression. These results provide a multi-scale description of the anatomical correlates of the mouse DMN.

INTRODUCTION

Large-scale brain networks support sensory perception, cognition, and motor output. Some networks, called resting state (rs) networks, have less well-defined functions and are characterized by temporally correlated intrinsic activity. The rs default mode network (DMN) emerges through rs functional magnetic resonance imaging (rsfMRI) as an anatomically distributed set of brain regions with low-frequency correlated activity in the absence of a goal-directed task (Raichle, 2015). DMN analogs have been identified in monkeys (Vincent et al., 2007), rats (Lu et al., 2012; Upadhyay et al., 2011), and mice (Gozzi and Schwarz, 2016; Grandjean et al., 2020; Sforazzini et al., 2014; Stafford et al., 2014). Conservation of this network across mammalian species suggests a central role in organizing healthy brain activity. Indeed, the DMN is particularly vulnerable in Alzheimer’s disease (AD), showing early accumulation of amyloid deposits and network dysfunction (Buckner et al., 2008; Greicius et al., 2004; Jones et al., 2016; Seeley et al., 2009). The DMN encompasses high-centrality hub regions (Buckner et al., 2008; Liska et al., 2015) that may serve as vulnerability points for psychiatric disorders such as autism and schizophrenia (van den Heuvel and Sporns, 2013; Menon, 2011).
rules of cortical connectivity across species (Goulas et al., 2019), including cell-class-specific projection patterns (Harris and Shepherd, 2015), hierarchical organization (Felleman and Van Essen, 1991; Harris et al., 2013), and modular network architecture with some very highly connected nodes (“hubs”: Coletta et al., 2020; van den Heuvel et al., 2016; Rubinov et al., 2015; Swanson et al., 2018; Zingg et al., 2014). Structural connectivity is also generally well correlated with functional connectivity in non-human species (Grandjean et al., 2017; Hutchison and Everington, 2012; Sethi et al., 2017), and comparisons within the DMN have revealed direct anatomical connections between areas (Andrews-Hanna et al., 2010; Coletta et al., 2020; Grandjean et al., 2017; Mantini et al., 2011; Stafford et al., 2014). However, the correspondence is not 1:1. Because brain regions participate in multiple, partially overlapping functional networks, multiplexing may instead be achieved at the level of cell populations or types.

Cross-species conservation of structural connectivity rules and rs networks suggests that we can gain relevant insights into the anatomy of human functional networks by studying animal models (Díaz-Parra et al., 2017; Stafford et al., 2014). Here we provide a detailed spatial description of the brain regions and connections in the mouse DMN. We registered a rsfMRI DMN mask with the fully annotated 3D Allen Mouse Common Coordinate Framework Reference Atlas version 3 (CCFv3; Wang et al., 2020), allowing integrated analyses of the DMN with regional and cell-type-specific anterograde axonal tracing experiments from the Allen Mouse Brain Connectivity Atlas (MCA; Harris et al., 2019; Oh et al., 2014). We found that voxels and regions within the DMN are preferentially connected to other DMN regions. Using Cre-defined tracing data, we show how axonal projections from layer 2/3 (L2/3) DMN neurons target other DMN regions, whereas L5 neurons target areas in and out of the DMN. Individual L5 neurons could send axonal branches to targets in and out of the DMN, broadcasting information to multiple downstream targets. Alternatively, specific types of projection neurons may preferentially target in- or out-DMN regions. To test these possibilities, we used a dual retrograde/anterograde intersectional viral tracing method (Gore et al., 2013) to label all axon branches from neurons defined by one target. We found strong evidence for in- and out-DMN projection types in the ventral RSP and further characterized them using single-cell transcriptomics.

RESULTS

Spatial Maps of the Mouse DMN at Voxel and Regional Levels

To identify the DMN, we performed blood-oxygen-level-dependent (BOLD) rsfMRI experiments on lightly anesthetized mice (Gutierrez-Barragan et al., 2019). We found five rs networks (“components”) using low-dimensional group independent component analysis (ICA). Four matched previously described networks (Sforazzini et al., 2014): (1) default mode, (2) somatomotor, (3) lateral cortical, and (4) hippocampal. The fifth component contained cerebellar signal and noise. We aligned ICA components 1–4 to the Allen CCFv3 (Figure 1A) after thresholding each component at a Z score of 1. The DMN component was also thresholded at a Z score of 1.7 to identify “core” DMN structures. Finally, we symmetrized each component to generate 3D masks registered to the Allen CCFv3 at 100-μm voxel resolution (Figures 1B, 1C, and S1A–S1C).

To define the anatomical structures of the DMN, we calculated the percentage of voxels within the CCFv3-aligned DMN mask that overlap with structures annotated in CCFv3. Starting at the major brain division level, we found that most (77.6%) of the voxels in the DMN belong to the isocortex. The rest of the mask voxels (22.3%) are in fiber tracts, the striatum, olfactory areas, and the mediodorsal thalamus (Figure 1D). The core DMN mask is even more restricted to the cortex (89.6%). Overlaps of the other three rs networks with major brain divisions are shown in Figures S1E–S1G. We performed the same calculations for a set of 316 regions brain-wide (i.e., the “summary structure set”; Table S2). We identified 36 isocortical areas with at least one voxel in the DMN mask and 25 areas in the core-DMN mask (Figure 1E). In the thalamus, containing 2% of the total DMN mask, specific structures included several midline nuclei. We chose to conservatively define regions inside the DMN (“in-DMN”) as those with more than 50% of their voxels in the core-DMN mask; only 15 regions in the isocortex passed this threshold (Figure 1E, black bars over thick dashed line). Of note, 4 cortical regions had more than 50% of their voxels inside the Z = 1 but not the Z = 1.7 DMN mask and were considered “out-DMN” regions: the posteromedial and rostrolateral visual areas (VISpm and VISrl) and dorsal and ventral agranular insular cortex (Ald and Alv). The core mask was used only to define DMN structures; subsequent analyses used the Z = 1 DMN mask.

We noted a strong resemblance between the spatial patterns of the DMN masks and two of six structural connectivity-based cortical modules we identified previously using a community detection algorithm (Harris et al., 2019). Structures in each module are more strongly connected with each other than with structures in other modules. Most in-DMN structures (n = 12 of 15) belong to prefrontal or medial modules (Figure 1E), and most regions belonging to these two modules were also defined as in-DMN structures. The DMN also included two regions of the somatosensory cortex (trunk and lower limb) and secondary motor cortex (MOs). Locations for all in-DMN regions in CCFv3 space are shown in Figure 1F. In summary, the DMN was anatomically defined most specifically by the voxel-resolution mask, alternatively by a set of 15 cortical regions, and approximately by the combination of the prefrontal and medial modules.

Preferential Region-to-Region Connectivity in the DMN

The assignment of most in-DMN regions to only two modules suggests that connections inside the DMN are stronger than connections made to cortical areas outside of the DMN. To quantify in-DMN preferential connectivity, we used 300 anterograde viral tracer experiments distributed across the cortex in wild-type (WT) mice from the MCA (Oh et al., 2014; Figure 2A). We also included injections in the cortical pan-excitatory Emx1-IRES-Cre line (Emx1) and the Rbp4-Cre_KL100 line (Rbp4) that labels L5 projection neurons because they project to the same cortical targets with connection strengths that are significantly correlated for matched source areas (Harris et al., 2019).
First we measured the strength of axonal projections inside and outside of the DMN mask at voxel-level resolution. For each experiment, we compared the fraction of the injection site in the DMN mask (injection DMN fraction) with the fraction of cortical projections in the DMN mask (projection DMN fraction). We observed a significant positive correlation between these measures (Pearson correlation \( r = 0.94, p < 0.001; \) Figure 2B).

It is not surprising to find that injections inside the DMN send a higher fraction of their projections to the DMN, given that the mask is spatially continuous (Figures 1B and 1C) and connection density decreases as a function of distance (Markov et al., 2014b; Oh et al., 2014; Ercsey-Ravasz et al., 2013). To estimate how much preferential intra-DMN connectivity can be explained by spatial proximity, we applied a general linear model (GLM) to derive a distance coefficient and a DMN coefficient for each experiment (Figures S2A and S2B). Distance coefficients were negative or close to zero and had a low inverse correlation with the injection DMN fraction (\( r = -0.16, p = 0.005; \) Figure S2C). DMN coefficients, however, were positively correlated with the injection DMN fraction (\( r = 0.60, p < 0.001; \) Figure S2D). Thus,

**Figure 1. Identification of DMN Structures**

A. Workflow for registering fMRI data to the Allen CCFv3. (1) Align the in-house fMRI template to CCFv3. (2) Apply the obtained transform to the ICA components. (3) Threshold at \( Z = 1 \) for all masks and \( Z = 1.7 \) for the DMN core mask. (4) Symmetrize along the midline. (5) Overlay CCFv3 region boundaries.

B. 3D image of the ICA DMN component registered to the CCFv3 template.

C. Serial cutaway images showing the DMN on coronal sections.

D. 3D views showing the major brain divisions in CCFv3 and pie charts showing the composition by major brain division for DMN and core DMN masks.

E. Percentage of voxels overlapping the DMN masks within all isocortex structures and selected structures in other major brain divisions. Colored boxes around isocortex structures indicate module affiliation from Harris et al. (2019).

F. 3D views show spatial locations of DMN regions colored by module (in E). Abbreviations in Table S1; see also Figure S1 and Tables S2 and S3.
DMN regions do send more projections to other DMN regions, even after accounting for distance.

To test whether the tendency for regions inside the DMN to send more projections inside that network is unique to the DMN or a more general property of rs networks, we also calculated mask and distance coefficients for the other rs networks. In all cases, injections inside each mask had more cortical projections inside that mask, and injections outside had more cortical projections outside the mask, even after accounting for distance (Figures S2E and S2F). This relationship held for hippocampal injections in the hippocampal network (Figure S2F). In fact, the correlation between injection and projection fractions was stronger for the other cortical networks than for the DMN, suggesting that it may be less preferentially connected, consistent with its polymodal nature (Buckner and Dunlop, 2019).

Next we tested whether preferential connectivity is also observed at the regional level by grouping the 300 isocortex injections into in- or out-DMN brain regions (Figure 1E). Like at the voxel-level, injections into in-DMN structures indeed had positive DMN coefficients, although a few were zero or negative (Figures S2G–S2I).
The DMN mask boundaries do not always align with anatomical boundaries (Figure 2K). Thus, across regions, the injection DMN fraction was more predictive of the projection DMN fraction than whether the source region containing the injection was itself an in-DMN structure (see colormaps for individual points in Figures 2I and 2J). For example, injections in the DMN portion of the MOs had more DMN projections than MOs injections located outside of the DMN (see the bimodal distribution in Figure 2I and Figures S2K–S2R). At a module level, most injections in prefrontal and medial modules had more than 50% of their cortical projections inside the DMN mask (Figure 2L). Conversely, injections in lateral and auditory modules had less than 50% of their cortical projections in the DMN. These results were consistent when accounting for distance (Figure S2J).

A final summary of the cortical regions and projections that intersect the core-DMN mask is provided in Table S2. These results confirm preferential direct connectivity between DMN regions and also suggest that the DMN covers functionally segregable subdivisions of anatomically defined structures.

**Preferential Connectivity between DMN Regions by Layer and Projection Cell Class**

Although DMN regions are more strongly connected to other DMN regions, they still project outside of the DMN. We hypothesized that anatomical cell types with distinct target specificities might selectively route information within or between networks. Excitatory neurons in the cortex are classified by projection targets and layers into three major classes: intratelencephalic (IT) in L2–L6, pyramidal tract (PT) in L5, and corticothalamic (CT) in L6 (Harris and Shepherd, 2015). Although many corticocortical projections originate from L2/3 and L5, the L5 IT class generally projects to a larger set of targets (Harris et al., 2019). Critically, cells in other layers project to a subset of the L5 cortical targets (Harris et al., 2019). Thus, one possibility is that connections to in- and out-DMN areas are made by L5 cells, whereas IT cells in other cortical layers project specifically to in-DMN targets.

To investigate how layer- and class-specific projection patterns relate to the DMN, we used a published set of spatially matched viral tracing experiments in 14 Cre lines selective for L2/3 (Cux2-IRES-Cre and Sepw1-Cre_NP39) and L5 IT (Rbp4 and Tlx3-Cre_PL56 (Tlx3; Figure 3), but results from L4 IT, L5 PT, and L6 CT experiments are provided (Figure S3). Of
note, Cre-dependent viral tracer injections in Rbp4 label L5 IT and L5 PT cells (Harris et al., 2019). We classify the Rbp4 data here as L5 IT because we focused exclusively on intracortical projections, and the PT class has relatively few (Harris and Shepherd, 2015). Cortical projections arising from Cre-defined neuron classes in the anteromedial visual cortex (VISam; a DMN region) highlight some layer-specific cortical projection patterns (Figure 3B). Notably, L2/3 projections visually overlap with L5 output but target fewer regions, particularly in the contralateral hemisphere (Harris et al., 2019; Figure 3B).

L2/3 and L5 IT cell classes had significant correlations between the injection and projection DMN fractions (Figure 3C, r = 0.91, 0.96, p < 0.001). Notably, inside the DMN mask, L2/3 had a significantly higher fraction of projections in the DMN than L5 IT (p = 0.007; Figure 3C, inset). The fraction of DMN projections was also significantly higher for L2/3 compared with L5 IT experiments for in-DMN but not out-DMN sources (p = 0.003; Figures 3D and 3E). In fact, almost all of the projections arising from L2/3 inside the DMN target other DMN regions (median = 0.91 compared with 0.73 for L5 IT projections). Because the DMN mask is symmetrical across hemispheres and there are fewer contralateral projections from L2/3 IT neurons, we speculated that the higher fraction of projections in the DMN from L2/3 might be simply explained by this difference. However, removing the contralateral projections from this analysis did not change the relative differences between groups (compare Figure 3C and Figures S3C–S3E with Figures S3F–S3H).

Injections located in prefrontal and medial modules had significantly more DMN projections from L2/3 compared with L5 IT cells (Figure 3F; p = 0.01 prefrontal, p = 0.04 medial). Because the DMN is mainly composed of these two modules, we thought that this difference may reflect an overall tendency for L2/3 IT cells to have a more specific intra-module projection pattern compared with L5 IT cells. We tested this by measuring the fraction of intra-module projections for other modules with n > 1 source region in our dataset. In all cases, L2/3 had a higher fraction of intra-module projections than L5 IT neurons (Figure 3G). These results show that DMN target regions receive input from L2/3 and L5 IT cells in DMN source regions but that projections outside the DMN mask, from DMN sources, arise predominantly from L5 IT cells.

**Preferential Connectivity between DMN Regions by Target-Defined Neuron Type**

Cre lines are useful for labeling broad cell classes, but many contain a mix of cell types based on transcriptomic, morphological, and physiological properties (Gouwens et al., 2019; Tasic et al., 2016, 2018). So, although, at a population level, L5 IT cells project to areas in and out of the DMN, subclasses could project to these targets in non-random combinations (Chen et al., 2016; Economo et al., 2016; Han et al., 2018). We hypothesized that L5 projection neuron types may co-exist in DMN regions that preferentially target in- or out-DMN regions. To test this, we used an intersectional viral tracing approach to label all collateral axons of target-defined (TD) projection cell types (Gore et al., 2013).

Our experimental approach is illustrated in Figures 4A and 4B. We injected a retrogradely transported canine adenovirus encoding Cre (CAV2-Cre; Hnasko et al., 2006; Soudais et al., 2001) into a “primary target” region and a Cre-dependent adenovirus-associated virus (AAV-FLEX-EGFP) into a “source” region in A175 Cre reporter mice that express nuclear-localized tdTomato (nls-tdTom; Daigle et al., 2018). Thus, neurons with axon terminals in the primary target express Cre following retrograde transport and are visible brain-wide as nls-tdT+. In the source, co-infected cells that express Cre (nls-tdT+) will also express EGFP, which fills the cell (including axons), allowing visualization of projections to the primary target and all other targets (“secondary targets”; Figure 4B). TD experiments are named according to their source with a subscript indicating their primary target; i.e., an ACAd<sub>RSP<sub>vi</sub></sub> experiment has an AAV injection into the anterior cingulate area, dorsal part (ACAd) and a CAV2-Cre injection into the ventral RSP cortex (RSPv), so all cells expressing EGFP in that experiment have cell bodies in the ACAd and project to the RSPv. In cases where CAV2-Cre infected two target regions equally, both are listed in the subscript (e.g., ACAD<sub>RSP<sub>v</sub>-RSP<sub>d</sub>)). Importantly, CAV2-Cre has a tropism bias toward L5 over L2/3 neurons (Chatterjee et al., 2018), but this bias is expected to be in our favor in these experiments (given the L5 IT focus).

In- and out-DMN primary target locations for a given source were chosen based on analyses of projections labeled in WT, Emx1, or Rbp4 mice. For example, L5 IT axons originating from ORBl project widely to multiple cortical targets (Figures 4C and S4A–S4C), including ACAd (in-DMN) and the anterolateral visual area (VISA; out-DMN). In this example, we paired a source injection in ORBl with CAV2-Cre in ACAd (ORBl<sub>ACAd</sub>; Figures 4D and S4D–S4F). ORBl<sub>ACAd</sub> cortical projections appeared to be a subset of the total ORBl L5 projection pattern (compare with Figure 4C); axons were densest in the rostral DMN in both hemispheres and also targeted lateral cortical areas outside of the DMN. An ORBl source injection paired with VISA<sub>al</sub> revealed a different subset of the ORBl L5 projections (Figures 4E and S4G–S4I). These results show that TD projection mapping can reveal intracortical projection neuron types with distinct multi-area targeting patterns.

We sought to identify TD experiments with “specific” (i.e., few secondary targets) as opposed to “broad” projection patterns (Figure 4B) based on the assumption that limited target patterns are more likely to be formed by a distinct subclass of neurons. Note that TD experiments have a smaller volume of infected cells than WT or Cre tracer experiments (Figure S5A) and so may be expected to contact fewer targets, particularly when the larger injections result in injection across borders into secondary sources. Thus, to be certain that each TD experiment could reasonably be expected to represent a subset of the WT projections, we only compared experiments that met four criteria: (1) the source structure was the same; (2) if the source structure was less than 60% of the injection volume in either experiment, then the structure containing the second-highest fraction of the source injection was also the same; (3) injection centroids were ≤ 800 μm apart; and (4) the injection spatial overlap (Dice coefficient) was more than 0.05. Using these criteria, we identified 10 sets of injection-matched cortical experiments comprised of at least one experiment per type: (1) TD, in-DMN target; (2) TD, out-DMN target; and (3) a WT, Emx1, or Rbp4 L5 experiment (STAR Methods). This curated set includes data for 10 cortical areas (Figure 4F) with 105 total experiments (63 TD and 42 WT) in 8
We used this rigorously curated dataset to test our hypothesis that different L5 projection neuron types in DMN regions target sets of in- or out-DMN regions. We again found significant correlations between the injection and projection DMN fractions (r = 0.94 TD in-target, 0.96 out-target, 0.95 matched WT, all p < 0.001; Figure 4G). Overall, there was no difference in the fraction of in-DMN projections for sources paired with in-DMN targets compared with out-DMN targets (Figures 4H and 4I). However, we were intrigued to observe considerable variability in the DMN projection fraction for in-DMN source experiments, particularly when paired with out-DMN targets (white boxplot in Figure 4I). Sources in the prefrontal module sent a high fraction of their projections to the DMN regardless of whether they were paired with an in-DMN or out-DMN target. In contrast, sources in the medial module had a significantly higher fraction of projections inside the DMN when paired with in-DMN compared with out-DMN targets (p < 0.05). The high fraction of DMN projections from prefrontal sources regardless of their primary target is consistent with a hub-like configuration of this polymodal region, whereas the higher in-DMN fraction for medial sources paired with in-DMN targets implies the presence of separate in-DMN- and out-DMN-projecting cell types in medial regions.

**Figure 4. Target-Defined Projection Mapping Differentiates In-DMN and Out-DMN Projections for Sources in the Medial Module**

(A and B) Experimental design and terminology. (A) In a target-defined (TD) experiment, a “source” injection with a Cre-dependent viral tracer (AAV-FLEX-EGFP) is paired with a “primary target” injection of a retrograde virus encoding Cre (CAV2-Cre). In the source, Cre mediates expression of EGFP in co-infected cells, allowing visualization of brain-wide projections, including “secondary targets.” Experiments are done in Ai75 mice so that all cells infected retrogradely with CAV2-Cre express nuclear tdTomato (tdT). (B) Schematic illustrating three hypothetical TD projection patterns from an in-DMN source paired with an in-DMN (purple) or out-DMN (green) target. Out-DMN TD cells are shown projecting only to other out-DMN regions. In-DMN TD cells may project to a specific (dark purple) or broad (light purple) set of in-DMN targets.

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**TD Regional Projection Patterns Vary by Source and Module**

TD experiments project to significantly fewer targets than their WT matches (n = 33 ± 17 versus 63 ± 21, p < 0.001, Student’s t test; Figure 5B), but even with the match criteria above, the size difference between WT and TD injection volumes remained a confound for quantitative comparisons (Figure 5C, left). We therefore constructed a model to identify TD experiments with projection patterns that differ from the WT. We

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(5 of 15) in-DMN and 2 out-DMN sources. Experiment IDs are provided in Table S3.
used a large set of WT replicates to predict the Spearman correlation ($r_s$) for two experiments based on the volume of the smaller injection in the pair, the distance between the two centroids, and the overlap between the two injection sites (Figure S5D; STAR Methods). We used this model to calculate the predicted $r_s$ for each WT-TD pair and then identified pairs below the 95% prediction interval as less correlated than expected for replicate injections (Figure S5D). We call these “low corr” pairs. Some source structures had a higher fraction of low corr pairs than others (e.g., ORBl, ACAv, and ORBvl; Figures 5A and S5E). Specific examples of WT-TD pairs are shown in Figures S5F–S5H. Many sources in our dataset, like ACAd, had 0 low corr pairs, whereas others, like VISp and RSPv, had an intermediate fraction (more examples in}

Figure 5. Quantitative Comparisons of TD and WT Injection-Matched Pairs
(A) Sources ranked by the fraction of low corr pairs (left; top axis, black circles; bottom axis, number of pairs). Right: mean $r_s$ (top axis) for low corr (white circles) and non-low corr (black circles) WT-TD pairs. The numbers of total pairs (left) and low corr pairs (right) are plotted on bottom axes in gray.
(B) Distribution of $r_s$ values for low corr pairs grouped by module. Boxplots show median and IQR. Whiskers extend to 1.5 x IQR. Statistical significance was determined using a one-way ANOVA followed by multiple t tests.
(C–K) Cortical projection images (C, F, and I) and coronal section serial two-photon tomography (STPT) images through the source (D, G, and J) and target (E, H, and K) injection sites for three experiments in the RSPv. Bars on the bottom of (E), (H), and (K) show the fraction of the CAV2-Cre injection site in each brain region. In-DMN PL and ACAd targets (C–H)) reveal midline-projecting patterns, whereas out-DMN VISl and VISp targets (I–K) reveal a visually projecting pattern.
(L) Overlay of the cortical projection images from (C), (F), and (I).
(M) Cortical projection image from a matched RSPv WT injection. Experiment IDs: RSPvPL, http://connectivity.brain-map.org/projection/experiment/cortical_map/5252523663; RSPvACAd, http://connectivity.brain-map.org/projection/experiment/cortical_map/56994687; RSPvWT, http://connectivity.brain-map.org/projection/experiment/cortical_map/112595376. Gray, DMN mask; Black, region boundaries.
(N) Projection strengths (log-transformed normalized projection volume [NPV]) to selected targets, plotted for each TD projection type.
(O) Projection strengths to all targets in the prefrontal, medial, and visual modules. Statistical significance was determined using a multi-way ANOVA followed by Tukey’s post hoc test for group comparison.
Axes in (N) and (O) are truncated at $10^{-2.5}$. See also Figures S5 and S6 and Table S5.
Figure S6). One of the VISp low corr pairs included the target VISl (VISpVISl), which labeled projections resembling an LI-LM-PM projection type observed using sequence-based connectivity mapping (Han et al., 2018). Notably, $r_s$ values were significantly lower for low corr pairs in the prefrontal and medial compared with the visual module and in the medial compared with the prefrontal module (Figure 5B; $p < 0.02$, one-way ANOVA followed by multiple t tests).

Two DMN-Related TD Projection Patterns in RSPv

The $r_s$ for low corr pairs in RSPv, a core-DMN region, were some of the smallest in the dataset (white points in Figure 5A, right). So we next asked whether these specific TD projection patterns relate to the DMN. We identified a midline-projecting pattern in RSPvPL and RSPvACAd experiments (Figures 5C–5H and S6). In contrast, projections from RSPvVISp and RSPvVISpVISp experiments had a different portion of the WT pathway labeled; i.e., a visually projecting pattern (Figures 5I–5K). These projection types contact distinct subsets of WT targets (Figures 5L and 5M); midline-projecting experiments primarily reach in-DMN targets and visually projecting experiments reach more out-DMN targets.

Although these two labeled pathways are mostly non-overlapping, some axons were detected in the same target regions. Quantitative comparison showed that midline-projecting experiments, particularly RSPvPL, had stronger connectivity to prefrontal targets compared with visually projecting RSPvVISp experiments (Figures 5N and 5O). Conversely, visually projecting RSPv cells had stronger projections to targets in the visual module than midline-projecting types (Figure 5O). Indeed, most visual areas received little to no input from midline-projecting types, with the exception of the posterior-lateral visual area (VISpl), to which nearly all experiments projected (Figure 5N).

Notably, we found the midline DMN-projecting pattern only in the ventral caudal RSPv (analogous to A29a,b in the rat; Sugar et al., 2011; Vogt and Paxinos, 2014; Figures S7A–S7D). Midline-projecting cells were also labeled in a ventral caudal RSPv TD injection paired with the VISpl and medial entorhinal cortex (ENTm) but not with the VISpl alone (Figure S7C versus S7D). Because of the known tropism bias of CAV2-Cre to L5 neurons, we confirmed that midline-projecting (RSPvACAd and RSPvVISp) and visually projecting (RSPVISp) cells were also labeled with another retrograde rabies virus, RVΔGL-Cre, without this bias (Chatterjee et al., 2018; Figures S7E–S7G). Critically, RVΔGL-Cre experiments labeled many more L5 than L2/3 cells (Figure S7H), indicating that L2/3 cells do not contribute in a major way to these two projection-types.

We noted that the midline-projecting RSPv injections appeared to label L5 cells located superficially to those in visual-projecting experiments (Figures 6A and 6B). CCF3 visualization placed most source cells from all experiments in L5, but midline-projecting experiments had some signal assigned to L2/3, and visually projecting experiments had slightly more signal assigned to L6 (Figure 6C). Small variances in registration precision might result in mis-assignment of voxels in deep L2/3 to L5 or vice versa. We therefore confirmed the sublayer locations of these projection types by comparing the distribution of tdT expression in 4 cortical layer-selective Cre driver lines crossed to the Ai14 reporter line (Figures 6D–6H) with the distribution of EGFP fluorescence levels inside the TD injection sites (Figure 6H, right). The peaks of the distributions were located ~40% (midline-projecting) and 60% (visually projecting) of the relative distance from the pia to the white matter, a range more like the L4/5 and L5 Cre lines (Scn1a and Rbp4) than the L2/3 (Cux2) or L6 (Ntsr1) line. These plots suggest that both projection types are indeed in L5 but that midline-projecting neurons are located in a sublayer above most of the visually projecting population.

We further validated the sublaminar locations of these projection types in the RSPv with data generated by mono-synaptic cell-type-specific rabies tracing experiments. We injected a Cre-dependent AAV (expressing the EnvA TVA receptor, tdT, and rabies glycoprotein), followed by an EnvA-pseudotyped, glycoprotein-deleted rabies virus expressing nuclear-localized EGFP into the prelimbic area (PL), ACAd, or VISp of Rbp4-Cre mice (Lo et al., 2019). RSPv L5 cells were labeled in all three cases. Cells projecting to the PL were indeed superficial to cells projecting to the VISp. ACAd projecting cells were intermingled or between PL- and VISp-projecting cells in these experiments (Figure 6I). We conclude that there are at least two projection types in RSPv L5: one that primarily projects to DMN targets (midline-projecting) and a second that primarily projects to visual targets (visually projecting).

Correspondence of RSPv Projection Types and Transcriptomic Types

To determine how the different RSPv projection types relate to transcriptomically defined cell types, we performed Retro-seq experiments (retrograde viral tracing combined with single cell sequencing; Tasic et al., 2018; Figure 7A). We injected CAV2-Cre or rAAVretro (Tervo et al., 2016) into the ACA or VISp in Cre reporter mice (Ai14, Ai75). We then isolated cells from the caudal part of the RSPv for single-cell transcriptomic profiling using SMART-Seq v.4 (Tasic et al., 2018). We mapped these cells into a recently developed comprehensive cell type taxonomy for the entire mouse isocortex and hippocampal formation (Yao et al., 2020) using gene expression profiles (Figure 7B; STAR Methods). Most midline-projecting cells mapped to a single cluster, 236_L3 RSP-ACA. Notably, many visually projecting cells also mapped to this cluster. We found other transcriptomic types among the visually projecting cells, including 255_L5 PT RSP-ACA (one ACA-projecting cell also mapped to this cluster). Cluster 236 is an unusual subclass. It strongly expresses two L4 marker genes, Scn1a and Rspo1, one of two L5 PT cell markers (Bcl6 but not Fam84b), and a pan-IT marker, Slc30a3. Yao et al. (2020) concluded that this subclass has an intermediate cell type identity between IT and PT. It was called L3 because of mixed-layer marker gene expression and because there is no anatomically defined L4 in the RSPv. Our results suggest that the location of these cells is more consistent with superficial L5.

Because genetically distinct cell types may exist within a single cluster (Kim et al., 2020), we further analyzed the sequencing results to look for differentially expressed genes (DEGs). First we
compared gene expression levels between all midline-projecting compared with all visually projecting cells in female mice only to avoid identifying sex-specific genes. We found three genes with higher expression in midline-projecting cells (Arc, Gne, and Rab15; Figure 7C). Next we compared only midline-projecting and visually projecting cells mapped to cluster 236, identifying five DEGs, all with higher expression in midline-projecting cells (Figure 7D). Notably, two of these genes, Arc and Gne, were identified in both comparisons.

The Allen Mouse Brain Atlas contains in situ hybridization (ISH) data for both of these genes, but, unfortunately, Gne mRNA detection is poor. Arc expression, however, appears to be stronger in the superficial part of L5, where the midline-projecting cells are found (Figure 7E). These results suggest Arc as a potential marker gene for the midline projection type.

Finally, we confirmed that visual-projecting RSPv VISp/VISl experiments are consistent with the PT classification. We observed labeled axons in the midbrain (Figure 7F) and thalamus (Figure 7G). In contrast, the midline-projecting types lacked subcortical projections, consistent with the IT cell class (Figures 7F and 7G).

**DISCUSSION**

**Anatomical Structures of the Mouse DMN**

The core DMN structures we identified here are all within the isocortex, in good agreement with previously published descriptions of rodents (Ash et al., 2016; Grandjean et al., 2017, 2020; Lu et al., 2012; Sforazzini et al., 2014; Stafford et al., 2014; Upadhyay et al., 2011; Zerbi et al., 2015). Hippocampal and retrohippocampal regions (including the entorhinal cortex) have been suggested in some studies to be part of the DMN (Lu et al., 2012; Upadhyay et al., 2011; Zerbi et al., 2015) but not others (Sforazzini et al., 2014; Stafford et al., 2014). These inconsistencies may be caused by magnetic field inhomogeneity in the rodent ear canal, which can limit reliable detection of these structures (Hsu et al., 2016; Lu et al., 2012), but may also reflect differences in the DMN detection strategies employed. We cross validated our ICA-based DMN selection by comparing the topography with seed-based probing of the anterior cingulate. In addition, a similar DMN topography has been described recently using non-correlational rsfMRI dynamic mapping (Gutierrez-Barragan et al., 2019). Imaging in
humans also suggests that other subcortical structures may belong in the DMN; e.g., thalamic regions (Alves et al., 2019). We also found that the DMN mask overlapped with some voxels in the mouse medial thalamus, which is highly interconnected with prefrontal cortical areas (Coletta et al., 2020; Harris et al., 2019).

The overlap of the DMN mask with the primary sensory areas, primary somatosensory area, trunk (SSp-tr) and primary somatosensory area, lower limb (SSp-ll) was somewhat surprising. However, a recent multi-site effort to map the mouse DMN at higher spatial resolution also revealed similar fringe involvement of primary sensory areas (Grandjean et al., 2020), and portions of the primary somatosensory cortex were included in the putative DMN identified by Stafford et al. (2014). The MOs is a relatively large cortical region, and its anatomical boundaries are not well-aligned with the DMN. Projections from the medial MOs, bordering the ACAd, are more consistent with the DMN than the lateral MOs. These results emphasize the importance of having a DMN map at a voxel level in CCFv3 space, enabling re-analyses with new or alternative anatomical parcelisation schemes.

Separation of the DMN regions into two modules is reminiscent of reports that the human DMN is composed of multiple interacting subnetworks (Andrews-Hanna et al., 2010; Braga and Buckner, 2017; Braga et al., 2019). However, direct comparisons of human and rodent structures are problematic because clear homologs may not exist; e.g., the precuneus, posterior cingulate areas 23 and 31 (Vann et al., 2009; Vogt and Paxinos, 2014), and the dorsolateral prefrontal cortex (Carlén, 2017). Some DMN regions do appear to be well conserved; e.g., the RSP, anterior cingulate, and medial prefrontal cortex (Carlén, 2017; Lu et al., 2012; Sforazzini et al., 2014; Stafford et al., 2014; Upadhyay et al., 2011; Zerbi et al., 2015). Recent cross-species probing of DMN dysfunction supports this notion; comparable DMN-centered alterations in connectivity were observed in mice and human subjects harboring autism-associated 16p11.2 chromosomal microdeletions (Bertero et al., 2018).
We expanded previous reports of preferential structural connectivity between mouse DMN regions (Grandjean et al., 2017; Stafford et al., 2014) by using tracer data from Cre driver lines (Harris et al., 2019) to test how intracortical projections from excitatory neuron classes in different cortical layers relate to the DMN. Notably, we found that L2/3 cells in the DMN primarily project inside the DMN, whereas L5 IT cells have in- and out-DMN projections. Using the TD approach, we further describe how cell types in L5 projecting to one in-DMN target are more likely to project to other in-DMN targets, but only for sources in the medial module. These results support the notion that specific projection neuron types underlie distinct functional network connectivity.

We observed that some cortical regions, such as the ACAd, have broadly distributed projections even from TD neuron populations, whereas other regions contain cell populations with more specific targeting patterns. This is perhaps not surprising, given that the ACAd has been reported to be a key integrative hub region for the mouse DMN (Coletta et al., 2020), but further suggests that network features like hubs may emerge at cell type and regional levels. Whether individual neurons within the ACAd project to most of ACAd’s targets or whether the specific set of targets tested here are shared among many projection types may be better addressed by ongoing efforts to reconstruct the complete morphology of single labeled neurons (Peng et al., 2020; Winnubst et al., 2019).

**Projection Neuron Types in the RSPv**

Midline-projecting in-DMN cells and visually projecting out-DMN cells in the RSPv are reproducibly separated by retrograde tracer injections in the ACA, PL, and Vi5Pl (midline-projecting) or Vi5Sp or Vi5l (visually projecting). Further, they can be distinguished anatomically based on subcortical projections (only visually projecting cells had subcortical projections) and soma location in cortical layers. However, cells from both projection types were assigned to the same transcriptomic cluster using a standard classifier based on expression levels of 3,188 genes. Using supervised analyses for DEGs among the two projection types, we identified a few potential marker genes for the midline-projection types, notably Gne and Arc. Future experiments combining retrograde viral labeling with ISH will be necessary to validate these findings.

Notably, midline-projecting cells were found in only one part of the RSPv, caudal to the corpus callosum splenium in the most ventral portion adjacent to the postsubiculum. This region of the RSPv is part of the medial subnetwork identified by Zingg et al. (2014), who noted its strong reciprocal connectivity with the anterior cingulate area, ventral part (ACAv); orbital area, medial part (ORBm); PL; and infralimbic area (ILA) and its connections with the ENTm and the subiculum. This network is particularly interesting with respect to the DMN because midline-projecting cells in the RSPv are ideally situated to convey hippocampal output from the subiculum to the medial prefrontal cortex. Alternating activation of midline-projecting and visually projecting cells in RSPv could act as a switch between rs DMN activity and visual processing networks. Functional characterization of these cells in various behavioral states would help clarify their role in the DMN.

**Relevance of Mouse to Human DMN Comparisons**

A unifying theory for cross-species comparisons of cortical DMN anatomy can be posed from analyses of spatial gradients in molecular, anatomical, and functional features across the primate cortex, which result in hierarchical ordering of regions from primary sensory to transmodal association areas (Buckner and D’Innocola, 2019; Buckner and Margulies, 2019; Margulies et al., 2016). Recent evidence suggests that a similar organization may be phylogenetically conserved in rodents (Coletta et al., 2020), where the dominant cortical gradient obtained from the structural and functional connectomes separates unimodal latero-cortical motor-sensory regions from transmodal components of the DMN, recapitulating the anatomical organization of the network we described here. Cortical hierarchies are also defined by laminar-based projection patterns into feedforward and feedback pathways (Felleman and Van Essen, 1991). In the mouse, we recently showed a shallow overall cortical hierarchy of areas. Notably, most of the DMN regions were at the top end of this purely anatomically based hierarchy. Furthermore, when the six cortical modules were ordered hierarchically using laminar-based rules, the prefrontal and medial modules were at the top (along with the lateral module), whereas somatomotor, visual, and auditory modules were at the bottom (Harris et al., 2019).

Our mesoscale data on cell class-specific connectivity provides new testable predictions about the anatomical basis of functional networks. For example, L2/3 neurons may play a unique role in supporting DMN cohesion, cross-hemisphere synchronization of rs networks, and intra-module communication, whereas subclasses of L5 neurons may be involved in coordination and switching across different brain-wide networks. The description of layer- and projection-specific cellular DMN components also provides potential inroads into understanding the selective vulnerability of the DMN to pathological processes in human diseases like AD, autism, and schizophrenia (Buckner et al., 2008; Fu et al., 2018).

**STAR METHODS**

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

Conceptualization, J.A.H., J.D.W., A.G., and H.Z.; Supervision, J.A.H., A.G., S.M., H.Z., L.N., P.A.G., and J.D.W.; Project Administration, W.W.; Investigation, Validation, Methodology, and Formal Analyses, J.D.W., A.L., K.E.H., P.B., A.W., P.A.G., N.G., J.E.K., A.H., P.R.N., T.N.N., C.T.J.v.V., E.G., O.F., M.N., A.M.H., N.D., K.A.S., B.L., B.T., S.M., A.G., and J.A.H.; Software, N.G., J.E.K., A.H., P.R.N., T.N.N., C.T.J.v.V., E.G., O.F., M.N., and A.G. The original draft was written by J.D.W. and J.A.H. with input from A.G. All co-authors reviewed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and Virus Strains** | | |
| AAV2/1.CAG.FLEX.EGFP.WPRE.bGH | UPenn Vector Core | Addgene AAV1; 51502 |
| CAV2-Cre | Viral Vector Production Unit at the Universitat Autonoma de Barcelona | N/A |
| prVdGL-4Cre, RVΔGL-Cre | Chatterjee et al., 2018 | Addgene plasmid; 98039 |
| rAAV2-retro-EF1α-Cre | Tervo et al., 2016 | N/A |
| AAV1-DIO-TVA66T-dTom-N2cG | Allen Institute | N/A |
| RabV EnvA-N2c-histone-EGFP | Lo et al., 2019 | N/A |
| **Critical Commercial Assays** | | |
| SMART-Seq v4 | Takara | Cat#634894 |
| **Deposited Data** | | |
| Allen Mouse Brain Connectivity Atlas | Oh et al., 2014, Harris et al., 2019 | http://connectivity.brain-map.org |
| mouse_CAPs_ds1_part1 | Gutierrez-Barragan et al., 2019, Mendeley Data, v1 | https://doi.org/10.17632/7y6xr753g4.1 |
| mouse_CAPs_ds1_part2 | Gutierrez-Barragan et al., 2019, Mendeley Data, v1 | https://doi.org/10.17632/r2w865c959.1 |
| **Experimental Models: Organisms/Strains** | | |
| Mouse: B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J, Ai14(RCL-tdT) | The Jackson Laboratory | JAX: 007914 |
| Mouse: B6.Cg-Gt(ROSA)26Sortm75.1(CAG-tdTomato*)Hze/J, Ai75(RCL-nT) | The Jackson Laboratory | JAX: 025106 |
| Mouse: C57BL/6J | The Jackson Laboratory | JAX: 000664 |
| **Software and Algorithms** | | |
| Python 3.7 ([https://www.python.org](https://www.python.org)) | Python Software Foundation | RRID:SCR_008394 |
| Anaconda | Anaconda | https://www.anaconda.com/ |
| NumPy ([https://numpy.org](https://numpy.org)) | NumPy Developers | RRID:SCR_008633 |
| pynrdrd | Maarten H. Everts and contributors | https://github.com/mhe/pynrdrd |
| pandas | NumFOCUS | https://pandas.pydata.org/ |
| hSpy | The HDF Group (THG) | http://www.hSpy.org/ |
| SciPy ([https://scipy.org](https://scipy.org)) | SciPy developers | RRID:SCR_008058 |
| Statsmodels ([https://www.statsmodels.org/stable/index.html](https://www.statsmodels.org/stable/index.html)) | Statsmodels developers | RRID:SCR_016074 |
| pg8000 | Mathieu Fenniak | https://github.com/tlocke/pg8000 |
| GIFT toolbox | Neuroimaging Tools & Resources Collaboratory (NITRC) | https://www.nitrc.org/projects/gift/ |
| ANTs package | Github | https://github.com/ANTsX/ANTs |
| STAR v2.5.3 | Github | https://github.com/alexdobin/STAR |
| R 4.0.1 ([https://www.r-project.org](https://www.r-project.org)) | The R Project for Statistical Computing | RRID:SCR_001905 |
| limma | Ritchie et al., 2015 | http://academic.oup.com/nar/article/43/7/e47/2414268/limma-powers-differential-expression-analyses-for |
| scrattch.hicat | Tasic et al., 2018; Yao et al., 2020 | https://github.com/AllenInstitute/scrattch.hicat |
| Analysis and visualization code for this paper | Github | https://github.com/AllenInstitute/DMN |
| **Other** | | |
| Allen Brain Reference Atlases | Wang et al., 2020 | http://atlas.brain-map.org/ |
RESOURCE AVAILABILITY

Lead Contact
Correspondence and requests for materials should be addressed to the Lead Contact, Julie A. Harris (jharris@cajalneuro.com).

Materials Availability
This study did not generate new unique reagents.

Data and Code Availability
- The accession numbers for the raw fMRI timeseries data used in this paper are: Mendeley Data: https://doi.org/10.17632/7y6xr753g4.1, https://doi.org/10.17632/r2w865c959.1, available at these links (Part #1 of the dataset, N = 20 mice, https://data.mendeley.com/datasets/7y6xr753g4/1; Part #2, N = 20 mice, https://data.mendeley.com/datasets/r2w865c959/1).
- fMRI masks in CCFv3 space are available for download in nearly raw raster data (nrrd) and Gnu Zipped Archive (gz) format at http://download.alleninstitute.org/publications/regional_layer_and_cell-specific_connectivity_of_the_mouse_default_mode_network/(DMN = component 0).
- Axonal tracing data for target-defined experiments (including high resolution images, segmentation masks, full source/target coverage, and informatically-derived projection weights) are available through the MCA data portal (http://connectivity.brain-map.org/; filter for Tracer Type: Target EGFP), and associated application programming interface (API) and software development kit (https://github.com/AllenInstitute/AllenSDK).
- Cortical projection images and high resolution image series for experiments shown in the figures are available online at http://connectivity.brain-map.org/projection/experiment/cortical_map/experiment_ID (experiment_ID provided in the figure legends).
- Full image series for the layer-selective Cre transgenic mouse driver lines crossed to the Ai14 reporter line used to draw the layer boundaries in Figure 7 are available at http://connectivity.brain-map.org/static/referencedata.
- The Arc in situ hybridization experiment shown in Figure 7 is available online at https://mouse.brain-map.org/experiment/show/74273120.
- Additional software for accessing the TD dataset and reproducing the analyses and figures in this publication are available at https://github.com/AllenInstitute/DMN.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
Experiments involving mice were approved by the Institutional Animal Care and Use Committees of the Allen Institute for Brain Science in accordance with NIH guidelines. Heterozygous Ai75 mice (RCL-nT; Jackson Laboratory #025106) on the C57BL/6J background were used for all experiments except retro-seq, where homozygous Ai14 mice were also used (Jackson Laboratory #007914). Tracer injections were performed in male and female mice age P57-P61, except for experiments in which the AAV injection was targeted by intrinsic signal imaging and the CAV2-Cre injection was coordinate-based (n = 10 experiments). These 10 mice received a CAV2-Cre injection and implanted headpost at age P38-P42 and a second, ISI guided AAV-CAG_FLEX-EGFP injection at P53-P61. Age and sex for all injected mice are provided in Tables S4 and S6. Mice were group-housed with a 12-h light–dark cycle. Food and water were provided ad libitum.

METHOD DETAILS

Functional MRI and DMN identification
Bold fMRI timeseries data were acquired from n = 40 mice under light sedation (0.7% halothane, and artificial ventilation). rsfMRI acquisition parameters are described in greater detail in Gutierrez-Barragan et al. (2019). Briefly, mice were anaesthetized with isoflurane (5% induction), intubated and artificially ventilated (2%, surgery). At the end of surgery, isoflurane was discontinued and substituted with halothane (0.75%). Functional data acquisition commenced 45 minutes after isoflurane cessation. Mean arterial blood pressure was recorded throughout imaging sessions. Arterial blood gases (pCO2 and pO2) were measured at the end of the functional time series to exclude non-physiological conditions.

All rsfMRI data were acquired with a 7.0 Tesla MRI scanner (Bruker Biospin, Ettlingen) using a 72 mm birdcage transmit coil, and a four-channel mouse brain for signal reception. Single-shot echo planar imaging (EPI) time series were acquired using an EPI sequence with the following parameters: TR/TE 1200/15 ms, flip angle 30°, matrix 100 x 100, field of view 2 x 2 cm², 18 coronal slices, slice thickness 0.50 mm, 500 volumes and a total rsfMRI acquisition time of 10 or 30 minutes, respectively.
To optimally register fMRI time-series to the 3D Allen Mouse Brain Common Coordinate Framework, v3 (CCFv3), we adopted the procedure recently described in Pagani et al. (2016). Briefly, fMRI time-series were first registered to an in-house T2-weighted mouse brain template with a spatial resolution of 0.1042 × 0.1042 × 0.5 mm³ (192 × 192 × 24 matrix; Sforazzini et al., 2014) using fsl-filt and 12 degree-of-freedom. A low component group ICA analysis was then performed with GIFT toolbox (https://www.nitrc.org/projects/gift/) in the coordinate system of the template. Previous studies showed that the use of low component ICA (N = 5) permits the identification of a DMN-like network in the mouse brain (Sforazzini et al., 2014). ICA analysis was run on demeaned data using the Infomax algorithm, with no autofill of data reduction values, and a brain mask to remove non-brain signal. ICASSO (1000 iterations) was used to ensure the stability of ICA components. All the other default parameters of GIFT (included PCA data reduction) were left unaltered. Independent components were scaled to z scores and thresholded at |Z| = 1, or |Z| = 1.7 as in Sforazzini et al. (2014). These thresholds exceed thresholding levels employed in previous rodent rsfMRI studies (Becerra et al., 2011; Hutchison et al., 2010; Jonckers et al., 2011; Liang et al., 2011). To maximize component orthogonality and minimize the contribution of between-network anti-correlation (Gutierrez-Barragan et al., 2019; Sforazzini et al., 2014), only positive ICA weights were considered.

The results of DMN-based identification of ICA were independently corroborated by comparing the maps obtained with seed-based probing of the anterior cingulate, a core hub component of the rodent DMN (Gozzi and Schwarz, 2016; Figure S1D). To this purpose, a 3 × 3 seed voxel placed on the anterior cingulate of spatially-registered time series and individual subject correlation maps were transformed to normally-distributed z scores using Fisher’s r-to-z transformation, before assessing group level connectivity distributions using one sample t tests. The resulting group map was thresholded at |T| = 8 (corresponding to uncorrected p < 0.00001, two-tailed test), and symmetrized along the sagittal midline. The T threshold employed maximizes spatial correlation (Spearman rho 0.73) and dice coefficient (0.75) between |Z| = 1 ICA and seed-based map.

Projection of in-house mouse brain template into Allen CCFv3 Reference Space

We projected the in-house MRI template (and co-registered ICA components) onto the CCFv3 using a combination of linear (affine) and non-linear (Syn) registrations using the ANTs package (antsRegistration command; Avants et al., 2011). Before the registration, we initially aligned images used images intensity (-r option) to ensure an initial rough alignment. For both transformations, the optimization was performed over five resolutions with a maximum of 100 iterations at the coarsest level. At the full resolution, 10 iterations were used for the affine registration, and 5 iterations were used for the non-linear registration. Smoothing and shrinking factors (7x5x5x3x1 and 9x7x5x3x1, respectively) were the same for both transformations. To enhance contrast, and hence help the registration, a slightly dilated brain mask was used. The gradient step was set to 0.1 for the affine registration, and to 0.15 for the Syn algorithm. In order to prevent non-realistic deformations, the spatial shifting in space for each iteration of the Syn algorithm was controlled by a smoothing factor (updateFieldVarianceInVoxelSpace = 3.0), whereas this was not the case for the total deformation (totalFieldVarianceInVoxelSpace = 0). For both the affine and the Syn algorithms, we adopted mutual information as similarity metric. The number of bins was set to 32, and values were sampled regularly in 50% of the voxels.

Assignment of functional network masks to CCFv3 voxels

The parameters obtained from registering the bias field corrected in-house template were used to linearly project the ICA components into CCFv3 (antsApplyTransforms). The N3 algorithm as provided by ANTs was used to perform bias field correction (N3BiasFieldCorrection: Shrinking factor was set to 1, 50 iterations and 4 fitting levels were used). Registration quality was assessed by qualitatively comparing a brain mask mapped from the coordinate system of the in-house template into the CCFv3 reference space and the Allen “root” label (whole brain mask). Since the cerebellum (coronal slices 1 to 114) was a region of no interest, it was removed from the both images. Functional network masks were symmetrized in CCFv3 space by shrinking to the minimum overlap between the two hemispheres.

Assignment of CCFv3 structures to fMRI masks

To determine which mouse brain structures were part of each ICA component in the fMRI dataset, we first computed the fraction of each fMRI map covered by each of the 12 structures in the CCFv3 ontology at the “coarse” level (structure set 2) plus “fiber tracts” by summing the number of voxels in the 100 μm resolution structure map that overlap with the fMRI mask and dividing by the total number of voxels in the fMRI mask. We further computed the overlap of the fMRI masks with each of the 316 “summary structures” from the Allen Reference Atlas (structure set 167587189) by summing the number of 100 μm voxels in each structure mask that overlapped with the fMRI mask and dividing by the total number of voxels in the structure. Selected structure overlaps with the DMN are presented in Figure 1E, and the overlap of all summary structures with the DMN and core masks are included in Table S2. The code for computing the overlap of summary structures with any of the fMRI masks is available at https://github.com/AllenInstitute/DMN.

Projection data analysis: DMN connectivity in WT experiments

We used 300 anterograde tracing experiments from the MCA with injections in the isocortex from WT C57BL/6J mice (n = 129), Emx1-IRES-Cre mice (Emx1, n = 66), and Rbp4-Cre_LK100 mice (Rbp4, n = 105). We previously showed that projections in Emx1 and Rbp4 mice are highly correlated with experiments in WT mice (Harris et al., 2019). This dataset can be viewed at http://connectivity.brain-map.org/ (Filter Source Structure(s): Isocortex, Filter Mouse Line: wild, Emx1-IRES-Cre, Rbp4-Cre_KL100). We used the 100μm grid volumes for this analysis: arrays of 100 μm voxels with values between 0 and 1 that indicate the density of
segmented pixels inside each voxel for voxels inside the injection polygon (injection grid) and outside the injection polygon (projection grid, see Oh et al., 2014). To quantify the injection DMN fraction, we first divided the sum of the gridded injection voxels inside the DMN mask by the sum of all injection voxels. We restricted our projection analysis to cortical projections by applying the isocortex mask to the DMN mask, then computed the projection DMN fraction by summing the gridded projection voxels inside the isocortex-DMN mask and dividing by the total number of projection voxels within the isocortex mask. Left hemisphere injections were flipped to the right hemisphere for visualization purposes. When grouping these experiments by source structure, experiments with injections that spanned multiple source regions were categorized as in- or out-DMN based on their primary source region, i.e., the region that contained the majority of the injection volume (Kuan et al., 2015).

**DMN Coefficient**

To control for the expected higher projection density in voxels near the injection site, we calculated the distance between the injection centroid and all other 100 μm voxels in the isocortex. To eliminate false positive signal, we first thresholded each voxel at 1.6x10^-4, which was the median signal per isocortex voxel in two experiments with failed AAV-FLEX-EGFP source injections (see Knox et al., 2019; Figure S1 for more information about segmentation errors and false positive signal). We added an epsilon value of 0.01 to the projection density in each voxel, corresponding to the 95th percentile of the false positive signal in the two experiments with failed AAV-FLEX-EGFP source injections. Each voxel was classified as in-DMN or out-DMN using the fMRI mask. We then used a general linear model to derive a “distance coefficient” ($\beta_{dist}$) and a “DMN coefficient” ($\beta_{dmn}$) for each experiment (Equation 1).

$$\log_{10}(\text{projection density} + \epsilon) = \beta_{dmn} \cdot \text{dmn}(\bar{x}) + \beta_{dist}(\bar{x}, \bar{x}_a) + \beta_{const}$$

Where

$$\text{dmn}(\bar{x}) = 1 \text{ for in, } 0 \text{ for out}$$

$$x = \text{voxels (100 μm)}$$

$$\bar{x}_a = \text{injection centroid}$$

$$\epsilon = 0.01$$

The correlation between the fraction of each injection that was inside the DMN and the DMN coefficient or the distance coefficient was calculated using ordinary least-squares regression.

**Projection data analysis: Matched Cre Injections**

Cre lines were evaluated for selective transgene expression in distinct cortical layers by examining available in situ hybridization or other reporter expression data (Harris et al., 2014; Gerfen et al., 2013; see also http://connectivity.brain-map.org/transgenic and http://www.gensat.org/cre.jsp). In addition to determining the cortical layer preference for Cre expressing neurons, we also classified them into one of three broad classes of projection neurons based on the major brain divisions targeted (IT, PT, CT). Projection cell class in each Cre-dependent viral tracer experiment was determined by manual classification and by hierarchical clustering of projection patterns as described previously(Harris et al., 2019). Based on this prior classification, the following Cre lines were used to identify layer-specific projections in this study (layer and class in parentheses): Emx1-IRES-Cre (L2-6 IT PT CT), Cux2-IRES-Cre (L2/3 IT), Sesp1-Cre_NP39 (L2/3 IT), Nr5a1-Cre (L4 IT), Scnn1a-Tg3-Cre (L4/5 IT), Rorb-IRES2-Cre (L4/5 IT), Rbp4-Cre_KL100 (L5 IT PT), Tlx3-Cre_PL56 (L5 IT), A930038C07Rik-Tg1-Cre (L5 PT), Chm2-Cre_OE25 (L5 PT), Efr3a-Cre_NO108 (L5 PT), Sim1-Cre_KJ18 (L5 PT), Ntsr1-Cre_GN220 (L6 CT), Syt6-Cre_KI148 (L6 CT). Additional details on these Cre lines are included in the first tab (“cortex”) of Table S1 from Harris et al. (2019) (first 15 rows). We curated this set of injection experiments, listed in the first tab of Extended Data Table 3 in Harris et al. (2019), so that all the injection experiments in each Rbp4 anchor group were either inside the DMN mask (injection DMN fraction > 0.5) or outside the DMN mask (injection DMN fraction < 0.5). In most sources, all experiments fell either inside or outside the DMN mask, but four source regions (MOs, SSp-bfd, VIspm, and VIsp) each contained at least one experiment from both categories. We eliminated two out-DMN MOs injections (477037203 and 141602484) and two in-DMN VIsp injections (300929973 and 272821309) and completely eliminated the VIspm group (Rbp4 anchor id = 485237081). Although barrel cortex (SSp-bfd) is not a DMN region, the matched group of injection experiments in SSp-bfd were mostly inside the DMN mask. We therefore also eliminated a single out-DMN SSp-bfd injection (178487444). One additional VIsp experiment in a Rbp4-Cre mouse was manually classified as a PT projection type rather than an IT type, so that experiment was also dropped (517072832). This left 350 experiments in 24 sources (experiments from all six VIsp anchor groups were combined into a single group). The distribution of experiments across DMN structures and layers was: L2/3: 18 in-DMN, 28 out-DMN; L4: 10 in-DMN, 33 out-DMN; L5: 26 in-DMN, 50 out-DMN; L5 PT: 35 in-DMN, 48 out-DMN; L6: 25 in-DMN, 36 out-DMN; all layers: 14 in-DMN, 27 out-DMN. Left hemisphere injections were flipped to the right hemisphere for visualization purposes.
Automated segmentation of EGFP signal was performed as previously described (Kuan et al., 2015) with minor modifications. When CAV injection annotation and segmentation logical measurements, location, context, and the normalized intensities of all three channels. Regions in which this additional clas-
used for registration. Therefore, a supervised decision tree classifier was used to filter out segmentation artifacts based on morpho-
CAV2-Cre injection site from the red channel into the green channel decreases the signal-to-noise ratio of the true fiber signal.
detecting EGFP-expressing fibers in the target-defined anterograde projection data, high intensity bleedthrough of nuclei in the
Coordinates (Franklin and Paxinos, 2012). Coordinates are reported as anterior–posterior (AP, referenced from bregma), medial–lateral (ML, distance from midline at bregma), and dorsal–ventral (DV, depth measured from the pial surface of the brain). Stereotaxic co-
ordinates (Franklin and Paxinos, 2012). Coordinates are reported as anterior–posterior (AP, referenced from bregma), medial–lateral (ML, distance from midline at bregma), and dorsal–ventral (DV, depth measured from the pial surface of the brain). Stereotaxic co-
neural viral injection experiments are provided in Table S6.
Many injections into visual areas were guided by sign maps derived from intrinsic signal imaging (ISI) through the skull as described in Harris et al. (2019); see also methods at http://help.brain-map.org/download/attachments/2818171/Connectivity_Overview.pdf. Sign maps for all ISI guided experiments are available in the online data portal as part of the cortical projection viewer.
All viruses were injected into the left hemisphere, but images were flipped to the right hemisphere in figures to facilitate comparison with wild-type experiments.
TD experiments used CAV2-Cre lot numbers 483, 738, and 990 with a titer of 1.0x10^{12} genome copies/ml obtained from the Viral Vector Production Unit at the Universitat Autonoma de Barcelona, produced from a vector enabling expression of Cre under control of the CMV promoter (Hnasko et al., 2006), paired with rAAV2/1-CAG-FLEX-EGFP-WPRE-bGH stock numbers V3900 and V5749 with a titer of 2.97x10^{13} and 1.34x10^{13} genome copies/ml (AAV-FLEX-EGFP) obtained from the Penn Vector Core. Three TD experi-
ments (indicated in text) used RV-ΔGL-Cre at 4.26 x 10^{10} genome copies/ml as a retrograde tracer instead of CAV2-Cre, and two retro-seq experiments used AAV2-retro-ΔF1α-Cre at 5.0x10^{12} genome copies/ml as a retrograde tracer. rAAV and AAV2-retro were injected using iontophoretic current injection, 3 or 5 μA for 5 minutes at each depth specified in the stereotaxic coordinates or at 0.3 and 0.6 mm for ISI targeted injections. CAV2-Cre and and rv-ΔGL-Cre were injected via pressure using a Nanoject II system using 100-200 nL for each depth. Monosynaptic rabies tracing experiments used the rAAV helper virus AAV1-DIO-TVA66T-dTom-N2cG with a titer between 3.60x10^{12} to 1.31x10^{13} genome copies/ml, produced from a vector enabling Cre-dependent expression of EnvA specific TVA receptor modified to have reduced affinity with EnvA to decrease spurious rabies virus infection, rabies glycoprotein (G), and tdTomato in the cytoplasm of Cre-expressing infected neurons, paired with a G-deleted, ASLV type A (EnvA) pseudo-
typed rabies virus expressing a nuclear GFP reporter (RabV EnvA-N2c-histone-EGFP), with a titer of 5.00x10^{9} genome copies/ml injected 21 ± 3 days after the initial rAAV helper virus injection (Lo et al., 2019; Reardon et al., 2016). Both viruses were produced at the Allen Institute (Osakada and Callaway, 2013).

Twenty-one days after virus injection (9 days from the rabies virus injection date for input mapping), mice were perfused with 4% paraformaldehyde (PFA), then brains were dissected and post-fixed in 4% PFA at room temperature for 3-6 hours, followed by over-
night at 4 °C. Brains were stored in PBS with 0.1% sodium azide before proceeding to serial two photon tomography (STPT) imaging using a TissueCyte 1000 system (TissueVision, Inc.) as previously described (Oh et al., 2014). Briefly, coronal images were acquired every 100 μm through the entire rostral-caudal extent of the brain with xy resolution of 0.35 μm/pixel. Images shown in figures were cropped, downsampled, and dynamic range-adjusted using Adobe Photoshop CS6. To convert red images to magenta, the red channel was duplicated into the blue channel using Adobe Photoshop CS6. Left hemisphere injections were flipped to the right hemisphere for visualization purposes.

Data Quality Control
There are 182 paired Cre-dependent anterograde AAV-FLEX-EGFP and retrograde CAV2-Cre tracer experiments available on the Allen Institute website (http://connectivity.brain-map.org/ filter by tracer type “Target EGFP”), of which 121 were included in this analysis. We excluded 11 experiments due to their source and target injection sites overlapping, and we only included experiments with cortical sources and targets. Twenty-six additional TD experiments with subcortical sources and twenty-five with subcortical targets are included in the online dataset but were not analyzed here. Replicate experiments were attempted for every source target pair, and 21 source-target pairs had n > 3 experiments with the same primary source and target. One additional experiment has not yet been released online but is partially accessible through the AllenSDK (RSPvVISp experiment # 868641659). Additional data for that experiment is included in the code repository at https://github.com/AllenInstitute/DMN. One experiment (571410278) had 74% of its injection assigned to VISpl and 15% to the postrhinal area (VISpor), but its projection pattern was more similar to VISpor than VISpl so its primary injection structure was manually changed to VISpor for the purpose of identifying injection matched experiments. A list of all 183 TD experiments with their metadata and reason for inclusion/exclusion are included in Table S4.

CAV injection annotation and segmentation
Automated segmentation of EGFP signal was performed as previously described (Kuan et al., 2015) with minor modifications. When detecting EGFP-expressing fibers in the target-defined anterograde projection data, high intensity bleedthrough of nuclei in the CAV2-Cre injection site from the red channel into the green channel decreases the signal-to-noise ratio of the true fiber signal. We were unable to decrease the fluorescence intensity of the red channel because the autofluorescence from this channel is used for registration. Therefore, a supervised decision tree classifier was used to filter out segmentation artifacts based on morpho-
logical measurements, location, context, and the normalized intensities of all three channels. Regions in which this additional classi-
ifier were used were called “dense red zones.”
We evaluated the performance of the segmentation algorithm inside and outside of dense red zones by comparing segmented projections to manually traced projection data. We selected two image planes from three experiments that had regions of no red (no dense red zone in ROI), mid-red (ROI was partially covered by dense red zone), and high red (ROI completely covered by dense red zone), eGFP signal was manually traced in all ROIs by an expert annotator (JDW). The manual and automated segmentation were compared by calculating the Dice Coefficient. Fewer of the manually traced axons were detected in the high red ROI than in the low-red ROI (p = 0.01). Therefore, for TD experiments, we report the fraction of projections inside the DMN mask after computationally removing EGFP segmentation in the CAV2-Cre injection polygon. For each experiment, the primary injection sites for the source (AAV-FLEX-EGFP) injection was determined as previously described (Oh et al., 2014). The target (CAV2-Cre) injection site was determined using the boundaries of a second polygon that was manually drawn around the densest nuclear tdTomato labeling. To classify a TD experiment as either an “in-DMN” or “out-DMN” target, we calculated the percent of the CAV2-Cre injection polygon that overlapped with the DMN mask and used a threshold of 50% (> 50% = “in-DMN targets,” < 50% = “out-DMN”).

Generation of a ground truth dataset for comparing replicate mesoscale viral tracing experiments

Quantitative comparisons between viral tracer experiments are challenging because a large amount of the variation between experiments can be attributed to small variations in injection location (Billeh et al., 2016). We previously used a distance threshold (≤ 500 μm) between injection centroids to identify spatially-matched anterograde tracing experiments (Harris et al., 2019). However, this criterion alone was not sufficiently rigorous for TD – WT comparisons, in large part because TD injections had a smaller volume than WT injections (Figure S5A). The smallest TD injections had infected area diameters of 100-150 μm, so it was possible for the centroid of a TD source injection to be less than 500 μm from a WT experiment without the two experiments having any actual spatial overlap. Similarly, the centroid of a large WT injection could be located relatively far from a small TD injection, but the two experiments could still have large spatial overlap.

To determine the most important criteria for matching two injection experiments, we started by manually checking 1568 TD – WT experiments pairs and rating their match quality by visual inspection using the cortical projection view (available at http://connectivity.brain-map.org/projection/experiment/cortical_map/experiment_ID) and individual image sections in the injection site for both experiments (ratings provided in Table S5). These ratings were used to choose thresholds for the percent of the injection volume contained in the primary injection structure, the distance between centroids, and the overlap between injection volumes based on the Sørensen-Dice Coefficient (Dice Coefficient, o, Equation 2) that included most well-matched injection experiments and excluded most poor matches.

\[
o = \frac{2|X \cap Y|}{|X| + |Y|}
\]

We then applied these thresholds to pairs of WT experiments matched by injection structure to identify 627 pairs of experiments in the isocortex that we considered replicates (n = 1-429 pairs per source, median = 5. The criteria for calling two experiments an injection match were:

1. The two experiments had the same primary injection structure (structure containing the highest fraction of the injection density, see Oh et al., 2014).
2. If the primary injection structure made up less than 60% of the injection volume in either experiment, the secondary injection structure was also the same in both experiments (n = 69 pairs in this category).
3. The two experiments had injection centroids located < 800 μm apart.
4. The Dice Coefficient was greater than 0.05.

Right hemisphere injections were flipped to the left hemisphere to calculate overlap and distance. Experiments meeting these criteria were called “injection-matched” to distinguish them from groups of experiments with the same source injection structure that were not necessarily spatially matched.

This group of “WT” experiments consisted of injections of anterograde AAV-EGFP tracer (rAAV2/1-hSyn-EGFP-WPRE-bGH) in WT C57BL/6 mice (n = 88 experiments), a Cre-dependent EGFP tracer (rAAV2/1-CAG-FLEX-EGFP-WPRE-bGH) in Emx1-ires-Cre and Rbp4-Cre_KL100 mice (Emx1: n = 26, Rbp4: n = 54 experiments), and a Cre-dependent synaptophysin-EGFP fusion protein tracer (rAAV2/1.pCAG.FLEX.synaptophysinEGFP.WPRE.bGH) in Emx1 and Rbp4 mice (Emx1: n = 27, Rbp4: n = 13 experiments) for a total of 208 experiments. This dataset contained at least one pair of injections in 27 of the 43 cortical regions. An additional seven cortical regions were represented as matched secondary sources in experiments with < 60% of the injection volume in the primary structure.

We included Cre-dependent tracer injection experiments done in Emx1 and Rbp4 transgenic mice in the dataset we call WT replicates because the projection patterns from cortical injection experiments in these two mouse lines have been previously shown to be highly correlated with injections of anterograde EGFP tracer in WT mice (Harris et al., 2019). However, we confirmed that there were no differences in correlations between experiments performed in WT mice and these two Cre lines for this dataset. We calculated the Spearman correlation (r_s) for each pair of experiments using the normalized projection volume (NPV) in ipsilateral and
contralateral cortical target structures. To minimize the contribution of false positives, NPV values were thresholded at log10(NPV) = -1.5 before calculating correlation coefficients (Harris et al., 2019).

The Spearman correlation did not differ for the three viruses (EGFP, Cre-dependent EGFP, and Cre-dependent SypEGFP), but transgenic line was a significant factor (p = 0.13 for virus, p < 0.0001 for transgenic line, two-way ANOVA). Post hoc testing showed that no transgenic line pairs had \( r_s \) values significantly lower than WT – WT pairs. However, \( r_s \) values for Emx1 – Emx1 experiment pairs were significantly higher than several other pairs of transgenic lines (Emx1 – Emx1 versus WT – WT; p = 0.02, WT – Rbp4: p = 0.001, Emx1 – Rbp4: p = 0.008). In addition, WT – Emx1 correlations were significantly higher than WT – Rbp4 correlations (p = 0.006). We did not exclude any of these pairs from the replicate dataset based on these statistics. For the full set of WT replicates used to construct the correlation model (all Cre line/virus combinations), \( r_s \) was 0.86 ± 0.07.

**Correlation model selection**

Even after applying our injection match criteria, injection volume, distance between centroids, and injection overlap were all significant factors that predicted the magnitude of the Spearman correlation (p < 0.001 for all, multi-way ANOVA). We fit a simple model that predicts the \( r_s \) between two experiments given these three factors using the Dice coefficient as a metric of overlap and residual sum of squares (RSS) as the optimality criterion. We tested several simple functions relating \( r_s \) to these factors. The relationship between the smaller injection volume and the Spearman was better fit by an exponential function compared to logarithmic (RSS = 2.92 logarithmic versus 2.80 exponential). The relationships between the centroid distance and Dice coefficient and \( r_s \) were both linear (RSS for distance = 3.10 linear, 3.18 exponential; RSS for Dice coefficient = 2.99 linear and 2.99 exponential). We started with the exponential model based on injection volume and tested whether it was improved by the addition of parameters for the Dice coefficient or distance. We derived \( p \) values for the additional parameters by performing an F-test on the sum of squared residuals for the two models. The addition of parameters for distance or Dice coefficient further improved the model (RSS with distance parameter = 2.73, p = 0.0001 versus reduced model, RSS with Dice coefficient parameter = 2.71, p = 5e-6 versus reduced model). Including both distance and Dice coefficient further improved the fit (RSS = 2.68, p = 0.0005 versus model based on size and distance). The final model is presented in Equation 3 and Figure S5D.

\[
p = -0.15 \times 10^{-0.00003d} + 0.04o + 0.88
\]

Where

\[
\begin{align*}
\text{rs} & = \text{Spearman’s Rho} \\
\nu & = \text{volume of the smaller injection} \\
d & = \text{distance between injection centroids in microns} \\
o & = \text{Dice Coefficient}
\end{align*}
\]

**Selection of wild-type matches for target-defined experiments**

Using the same injection match criteria as we used for constructing the control dataset, we identified 586 pairs of matched TD and WT experiments, including at least one matched WT for all except 11 TD injections. The WT experiment matches consisted of injections of anterograde EGFP tracer (rAAV2/1-hSyn-EGFP-WPRE-bGH) in WT C57BL/6 mice (n = 49 experiments), Cre-dependent EGFP tracer (rAAV2/1-CAG-FLEX-EGFP-WPRE-bG) in Emx1-IRES-Cre and Rbp4-Cre_KL100 mice (n = 18 and n = 32 experiments), and a Cre-dependent synaptophysin-EGFP fusion protein tracer (rAAV2/1.pCAG.FLEX.synaptophysinEGFP.WPRE.bGH) in Emx1-IRES-Cre and Rbp4-Cre_KL100 mice (n = 19 and n = 10 experiments). 108 of 128 WT experiments matched to TD injections had also been used to fit the model. Note that the model did not depend on source identity, so six sources had at least one WT – TD match but no WT – WT matches: ILA, ORBm, VIsa, RSPd, ECT, and VIss. The set of WT-TD pairs had lower \( r_s \) values than WT-WT pairs (WT-WT: 0.86 ± 0.07, WT-TD: 0.78 ± 0.10, p < 0.001, Student’s t test). Observed \( r_s \) values were outside the 95% prediction interval of the model in 21% of all WT-TD pairs (43 pairs above, 80 pairs below). There was no relationship between the number of pairs tested in a source region and the fraction of low corr pairs observed in that source (r = 0.08, p = 0.7). Low corr pairs were relatively common in the prefrontal module; 5 of 7 sampled sources had at least one. However, medial, somatomotor, and visual modules had only 4 sources combined with low corr pairs out of 14 sampled (RSPv, MOs, and lateral (VIs) and primary (VIp) visual cortex). Low corr pairs are indicated in tab 3 of Table S5.

**Identifying matched experiment sets**

We used the same criteria to identify the largest groups of injection matched TD experiments that contained at least one experiment with an in-DMN target and one with an out-DMN target. Using the injection match criteria, we also identified all the WT experiments
that matched with every TD experiment in each set, allowing one mismatch for each WT experiment (i.e., a WT experiment could match with every TD experiment in a group except one and still be included in a “set”). The experiment ids in each of the ten injection matched experiment groups are presented in Table S4.

Measurement of cell depth distributions
Four cortical layer-selective Cre driver lines were crossed to the Ai14 reporter line, imaged with STPT and registered to CCFv3 (Wang et al., 2020): L2/3 (Cux2-CreERT2), L4/5 (Scnn1a-Tg3-Cre), L5 (Rbp4-Cre_KL100), and L6 (Ntsr1-Cre_GN220, projections in L5). We used the cortical streamlines in RSPv from CCFv3, which represent the shortest orthogonal path from the outer to inner cortical surface (Wang et al., 2020), to plot the distribution of fluorescent intensity across cortical depth in RSPv (Figure 6H, left).

Single-cell RNA-sequencing
Cells were collected by microdissection of RSPv from brains of mice with retrograde label. Single-cell suspensions were created and cells were sorted using fluorescence activated cell sorting (FACS). FACS gates were selective for cells with tdTomato-positive label. In total, 239 cells were collected from 6 mice (n = 3 ACA, n = 3 VISp, metadata provided in Table S6). Single cells were sorted into individual wells of 8-well PCR strips containing lysis buffer from the SMART-Seq v4 kit with RNase inhibitor (0.17 U/μl), immediately frozen on dry ice, and stored at −80°C. Cells were later processed for single cell RNA-seq using SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Takara Cat# 634894) as previously described (Tasic et al., 2018; Yao et al., 2020).

Sequence alignment was performed using STAR v2.5.3 (Dobin et al., 2013) in the two-pass mode. PCR duplicates were masked and removed using STAR option ‘bamRemoveDuplicates’. Only uniquely aligned reads were used for gene quantification. To identify their cell types, sequenced cells were mapped to the mouse cortex and hippocampus taxonomy (Yao et al., 2020). The median gene expression for each cell type within the taxonomy was calculated using the SMART-Seq v4 dataset as reference. Cells were mapped by computing the correlation of gene expression of an individual cell with the median gene expression for each cell type. To estimate the robustness of mapping, mapping was repeated 100 times, each time using 80% of randomly sampled genes, and the probability for each cell to map to every reference cluster was computed. The cell type with the highest probability of mapping was chosen as the corresponding cell type of that cell. Cluster mapping for all experiments is provided in Table S6.

Differential gene expression analysis between RSPv to ACA and RSPv to VISp projecting neurons were performed using the R package limma (Ritchie et al., 2015). Significantly differentially expressed genes were defined as having > 1.5-fold change and a Benjamin-Hochberg corrected P value < 0.05. Gene expression distributions of cells within a cluster or projection pattern were visualized using violin plots and heatmaps. To avoid detecting sex-specific genes, we only included cells isolated from female mice, as all ACA injections were performed in female mice only (VISp: n = 1 mouse, 42 cells; ACA: n = 3 mice, 58 cells).

QUANTIFICATION AND STATISTICAL ANALYSIS
Ranges of values are reported as mean ± standard deviation. Statistical details can be found in the results, figure legends, and STAR Methods sections. n generally represents the number of animals, except for the RNA sequencing data where n refers to the number of cells. Correlation coefficients reported as “r” refer to the Pearson correlation, and as “rs” refer to the Spearman correlation. Unless otherwise indicated, p values were computed using multi-way ANOVA with type II sum-of-squares and Tukey’s post hoc test for factors that were significant in the ANOVA. Significance was established using a p value of 0.05. When multiple t tests were performed for post hoc analyses, we used the Benjamini/Hochberg correction for multiple comparisons.
Supplemental information

Regional, Layer, and Cell-Type-Specific Connectivity of the Mouse Default Mode Network

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Figure S1 related to Figure 1. Overlap of functional networks with major brain divisions

(A-C) Additional components identified with independent component analysis (ICA) on resting state fMRI data. (D) An alternative DMN component generated with a seed pixel in ACA resembles the ICA map in Figure 1B,C. (E-G) Pie charts showing the composition of each component in terms of major brain divisions. Abbreviations: TH: thalamus, MB: midbrain, OLF: olfactory areas, CTXsp: cortical subplate, HPF: hippocampal formation, STR: striatum, PAL: pallidum.
Figure S2 related to Figure 2. Preferential DMN connectivity independent of distance and region boundaries

(A,B) Cortical projections (top) and projection density per isocortex voxel (bottom) plotted for in-DMN voxels (cyan) and out-DMN voxels (green) in one in-DMN (A) and one out-DMN (B) experiment. Lines on bottom plots show the model fit for in-DMN voxels (cyan) and out-DMN voxels (green). The magnitude and sign of the DMN coefficient is reflected in the distance between the in-DMN and out-DMN fit lines, and the magnitude of the distance coefficient by the slope of the lines. Experiment IDs: 125833030 (A), 180436360 (B). (C) Distance coefficient and (D) DMN coefficient for each of the 300 injection experiments in WT, Emx1-Cre, and Rbp4-Cre mice from the MCA. r = Pearson correlation. Experiments shown in A and B are labeled on the graphs in C and D (green and cyan borders). (E,F) Coefficients for the projections of the same 300 experiments in the lateral cortical and somatomotor masks (E) and hippocampal mask (F) as a function of injection mask fraction. (G) DMN coefficients for each of the 300 WT, Emx1, and Rbp4 experiments grouped by source structure. (H) Top down cortical projection map showing the location of these experiments colored by their DMN coefficient. (I,J) The same points shown in g grouped by in-DMN and out-DMN regions (I) and module (J). (K) Cortical surface map showing the location of the 18 injection experiments in secondary motor cortex (MOs) in WT, Emx1-Cre, and Rbp4-Cre mice from the MCA, colored by the fraction of cortical projections inside the DMN mask for each experiment. The boundary of the DMN and core masks are shown in gray and cortical structure boundaries in black. (L) Coronal view of the experiments in K overlaid on a maximum intensity projection of the corresponding portion of the average template brain, colored as in K. DMN and core masks are shown in gray, experiments shown in M and N are labeled. (M,N) Cortical projection images showing two example MOs injections. Asterisks indicate the approximate injection centroid. In-DMN projections are pseudocolored cyan and out-DMN projections green. Experiment IDs: 141603190 (C), 585025284 (D). Dashed lines show the rostral-caudal position of panels O-R. (O,P) Section through the center of the injection site showing segmented injection pixels (green) overlaid on the corresponding virtual coronal section from the CCFv3 template. (Q,R) Section at the approximate rostral-caudal position with the highest projection density showing segmented projection pixels (green) overlaid on the corresponding CCFv3 virtual template section. (O-R) DMN masks shown in light gray.
Figure S3 related to Figure 3. L4, L6, and L5 PT neurons have mainly local projections. (A) Location of matched injection experiments in L4 selective Cre lines (magenta, n=43), L5 PT selective Cre lines (blue, n=83), and L6 selective Cre driver lines (yellow, n=61), see Methods. Red circle indicates the injection centroids belonging to the anteromedial visual cortex (VISam) group; examples shown in B. The boundary of the DMN is shown in gray and cortical structure boundaries in black. (B) Cortical projections of experiments from the VISam group in a Scnn1a mouse line selective for L4 neurons (left), an Efr3a mouse line selective for L5 PT neurons (center), and an Ntsr1 mouse line selective for L6 neurons (right). Experiment IDs: Scnn1a 268038969, Efr3a 309515141, Ntsr1 156671933. (C) Scatterplot and linear fit for the fraction of cortical projections inside the DMN as a function of the fraction of the injection inside the DMN for each of the 187 cortical injections shown in A. Inset shows the projection DMN fraction for injections binned by the fraction of the injection polygon inside the DMN mask. (D) Distance coefficient, and (E) DMN coefficient plotted as a function of injection DMN fraction for the set of layer-selective experiments shown in Figure 3A and panel A. Boxplots show the same points binned by the fraction of injection polygon inside the DMN mask. (F) The fraction of ipsilateral projections inside the DMN as a function of the fraction of the injection inside the DMN. Inset shows the fraction of DMN projections for injections binned by the fraction of the injection polygon inside the DMN mask. The relative values for the different layer selective Cre lines are not different from the fraction of ipsilateral and contralateral projections inside the DMN (compare with C and Figure 3C). (G) The relationship between the ipsilateral distance coefficient and the fraction of an injection that is inside the DMN. The relative order of the Cre lines is the same as for ipsilateral and contralateral projections (D). (H) The relationship between the DMN coefficient for ipsilateral projections and the fraction of an injection experiment that is inside the DMN. The relative order of the Cre lines is the same as for ipsilateral and contralateral projections (E).
Figure S4 related to Figure 4. High magnification images of target-defined tracer experiments

(A) An ORBl injection in an Rbp4-Cre mouse. The injection site is saturated in this image so axons can be resolved. Projections from this experiment are visible in ACAd (B) and VISal (C). (D) An ORBl<sub>ACAd</sub> source injection (in-DMN target) showing cells coinfectected with both viruses (green), and nuclei from cells infected with only CAV2-Cre (magenta). Projections from the ORBl<sub>ACAd</sub> cells are shown inside the CAV2-Cre injection site (E, primary target) and in VISal (F, secondary target). (G) An ORBl<sub>VISal</sub> source injection (out-DMN target). Projections from ORBl<sub>VISal</sub> cells are shown in ACAd (H, secondary target) and VISal (I, primary target). Experiment IDs: ORBl<sub>Rbp4</sub> 156741826, ORBl<sub>ACAd</sub> 571816813, ORBl<sub>VISal</sub> 601804603. All sections are coronal. Arrowheads point to axons. Scale=500 μm, identical for vertically-aligned images.
Figure S5 related to Figure 5. Quantitative characterization of target-defined experiments

(A) Injection volumes in TD experiments are significantly smaller than injection volumes in WT, Emx1-Cre, and Rbp4-Cre experiments. (B) Number of brain-wide targets with labeled axons (thresholded at log-transformed normalized projection volume $> 10^{-1.5}$) in TD compared to their matched WT experiments. Thick black line: mean and standard deviation. (C) Injection volume (left), distance between injection centroids (center), and Dice overlap (right) for WT-WT experiment pairs (black and gray) and TD experiments (white). (D) Predicted (black line) and actual (points) Spearman correlation for each WT-WT (black), and WT-TD (white) injection-matched pair plotted as a function of the volume of the smaller injection in each pair. Gray lines show the 95% prediction interval. (E) Spearman’s Rho ($r_s$) for WT – WT injection matched pairs (gray boxplots) and WT – TD injection matched pairs (white points) plotted by source. Black borders indicate “low corr” pairs ($r_s$ below the 95% prediction interval). (F-H) Cortical projection images of matched WT (left) and TD (right) experiments with injections in ACAd (F, not low corr), VIs (G, low corr), and RSPv (H, low corr). Asterisks indicate the approximate centroid of each AAV source injection. Dashed magenta circles indicate the approximate area of the CAV2-Cre target injection. Experiment IDs: ACAd$_{WT}$ 146593590, ACAd$_{RSPagl}$ 607059419, VIs$_{WT}$ 100141219, VIs$_{VISl}$ 515920693, RSPv$_{WT}$ 112595376, RSPv$_{PL}$ 623838656. Arrowheads in the center panel show projections in VISl and VISal in the WT experiment that are absent in the TD experiment. Additional examples of TD experiments with ACAd and VIs source injections are presented in Figure S6.
Figure S6 related to Figures 5 and S5. Additional examples of TD source injections in ACAd and VISp. (A-D) Cortical projection views for an ACAd injection in a WT mouse (A), a L5-selective Cre driver mouse (Tlx3, B), and two TD experiments (C,D). (E-H) Single section image at the approximate center of each injection site. (I-L) One coronal section from each experiment. Boxed insets show the area enlarged in (M-P, contralateral) and (Q-T, ipsilateral). (U) Overlay of the top down cortical projections from the two TD experiments in C and D. Arrows in B,C,D,U indicate the only notable difference between these experiments: contralateral projections in the ACAd_RSPagl experiment. (V) r_s values for the four ACAd experiments shown here. Distance is given in parentheses. Scale = 500 µm. Experiment IDs: ACAd_WT_146593590, ACAd_Tlx3_293432575, ACAd_RSPagl_607059419, ACAd_MOp_475829896. (W) Normalized Projection Volume (NPV) in six visual and medial structures for three VISp TD experiments (gray), and for the single VISp experiment shown in Figure S4f (black). It is possible that pairing a similar VISp injection with CAV-Cre in a different VISl location would result in a different projection motif given retinotopic organization. (X) NPV in six visual and medial structures for 16 TD injections in VISp (gray). The VISp projection pattern (blue) resembles the VISp pattern in panel a, while the VISp (purple) projections had a different pattern with strong projections in VISli, VISi, VISal, and VISpm and no projections to VISam and VISrl. VISp (orange) projections had yet another pattern, with projections to all the visual areas included here, and strongest in VISrl and VISal. (Y-a) Cortical projection views (left) and sign maps (right) for the VISp (Y), VISp_Audp (Z), and VISp_Ssp-bfd (a) experiments shown in panel w. Asterisk indicates the approximate location of the AAV source injection centroid and dashed circles indicate the approximate area of the CAV2-Cre target injection. Experiment IDs: VISp_RSPagl_501787135, VISp_Audp_501837158, VISp_Ssp-bfd_539323512. The location of the injection centroid for the VISp injection relative to the sign map shows that this source injection was located in a different part of VISp than the VISp and VISp_RSPagl injections, more rostral and lateral. These subtle differences in source location and their correlation to different projection patterns emphasize the importance of careful experiment matching for quantitative comparisons between TD experiments.
Figure S7 related to Figure 5. Midline-projecting RSPv cells are only found in caudal, ventral parts of retrosplenial cortex, and can be labeled with either CAV2-Cre or RV-ΔGL-Cre target injections. (A-D) TD experiments with CAV2-Cre virus target injections. top: Cortical projections. Asterisk indicates the approximate centroid of the source injection and magenta circles indicate the approximate location of the target injection. middle: Single image section from the approximate center of the source injection site showing the location of EGFP-expressing cell bodies for cells that were infected with both viruses. bottom: Single image through the approximate center of the target injection site. Bars on the bottom of the images show the regions in each CAV2-Cre injection site as the fraction of the injection polygon that overlapped with each region. (A) An RSPv ACAd TD injection with a source in the rostral portion of RSPv does not have a midline-projecting pattern. (B) an RSPv ACAd TD injection with a source in the dorsal portion of caudal RSPv does not have a midline-projecting pattern. Arrowheads in a,b point to more dense axons in ACAd than ACAv. (C) Midline-projecting RSPv cells can be labeled with a target CAV2-Cre injection in VISpl. (D) A different RSPv VISpl TD experiment did not label midline-projecting cells. Experiment IDs: RSPv ACAd (A, rostral) 475830603, RSPv ACAd (B, dorsal) 571647261, RSPv VISpl (C) 592724077, RSPv VISpl (D) 666090944. (E-G) TD experiments with RV-ΔGL-Cre target injections. top: cortical projections. Asterisk indicates the approximate centroid of the source injection and magenta circles indicate the approximate location of the target injection. middle: Single image section from the approximate center of the source injection site showing the location of EGFP-expressing cell bodies for cells that were infected with both viruses. bottom: Single image through the approximate center of the target injection site. (E) A RSPv ACAd TD experiment with a midline-projecting pattern. (F) A RSPv VISp experiment with a visual-projecting pattern. (G) A RSPv VISpl experiment with a midline-projecting pattern. e-g Experimental data available through the AllenSDK. Experiment IDs: RSPv ACAd 605112318, RSPv VISp 595890081, RSPv VISpl 595261714. (H) Layer composition for each of the three TD source injections with RV-ΔGL-Cre as the target virus determined by registration to CCFv3. Scale = 500 µm.
| Abbreviation        | Full Name                                                                 |
|---------------------|---------------------------------------------------------------------------|
| AAV-FLEX-EGFP       | adeno-associated virus containing a flip-excision switch around the EGFP coding sequence driven by the CAG promoter |
| AD                  | Alzheimer's disease                                                        |
| BOLD                | blood-oxygen-level dependent                                               |
| CAV2-Cre            | canine adenovirus encoding Cre recombinase                                  |
| CCFv3               | Common Coordinate Framework version 3                                      |
| CT                  | corticothalamic (cell type)                                                |
| DEG                 | differentially expressed genes                                             |
| DMN                 | default mode network                                                       |
| Emx1                | Emx1-IRES-Cre mouse line                                                   |
| EPI                 | echo planar imaging                                                        |
| FACS                | fluorescence-activated cell sorting                                        |
| fMRI                | functional magnetic resonance imaging                                      |
| GLM                 | general linear model                                                       |
| ICA                 | independent component analysis                                             |
| ISH                 | In situ hybridization                                                      |
| IT                  | intratelencephalic (cell type)                                             |
| IQR                 | Interquartile range                                                        |
| L[#]                | cortical layer (e.g. L1, L2/3, L4, L5, L6)                                 |
| MCA                 | (Allen) Mouse Brain Connectivity Atlas                                     |
| module              | group of brain regions identified using a community detection algorithm     |
| network             | group of interconnected neurons or brain regions                           |
| nls-tdT             | nuclear-localized td (tandem-dimer) Tomato fluorescent protein             |
| PT                  | pyramidal tract (cell type)                                                |
| r                   | Pearson correlation                                                        |
| rs                  | Spearman correlation                                                       |
| Rbp4                | Rbp4-Cre_KL100 mouse line                                                  |
| ROI                 | region of interest                                                         |
| rs                  | resting state                                                              |
| RSS                 | residual sum of squares                                                    |
| scRNA-seq           | single cell RNA sequencing                                                  |
| SSV4                | SMART-Seq v4                                                               |
| STPT                | serial two photon tomography                                               |
| TD                  | target-defined                                                             |
| WT                  | wild type mice                                                             |

### Brain Regions

| Structure Abbreviation | Structure Name                      | Cortical Module |
|------------------------|-------------------------------------|-----------------|
| Isocortex              | Isocortex                           |                 |
| ACAd                   | Anterior cingulate area, dorsal part| Prefrontal      |
| ACAv                   | Anterior cingulate area, ventral part| Prefrontal     |
| PL                     | Prelimbic area                      | Prefrontal      |
| Abbreviation | Description                           | Region       |
|--------------|---------------------------------------|--------------|
| ILA          | Infralimbic area                      | Prefrontal   |
| ORBl         | Orbital area, lateral part            | Prefrontal   |
| ORBm         | Orbital area, medial part             | Prefrontal   |
| ORBvl        | Orbital area, ventrolateral part      | Prefrontal   |
| VIa          | Anterior area                         | Medial       |
| VIam         | Anteromedial visual area              | Medial       |
| RSPagl       | Retrosplenial area, lateral agranular part | Medial     |
| RSPd         | Retrosplenial area, dorsal part       | Medial       |
| RSpv         | Retrosplenial area, ventral part      | Medial       |
| SS-p-tr      | Primary somatosensory area, trunk    | Somatomotor  |
| SS-p-l       | Primary somatosensory area, lower limb | Somatomotor  |
| MOs          | Secondary motor area                 | Somatomotor  |
| SSs          | Supplemental somatosensory area       | Somatomotor  |
| SS-p-bfd     | Primary somatosensory area, barrel field | Somatomotor  |
| SS-p-u       | Primary somatosensory area, upper limb | Somatomotor  |
| SS-p-u       | Primary somatosensory area, unassigned | Somatomotor  |
| SS-p-n       | Primary somatosensory area, nose     | Somatomotor  |
| SS-p-m       | Primary somatosensory area, mouth    | Somatomotor  |
| MOp          | Primary motor area                   | Somatomotor  |
| FRP          | Frontal pole, cerebral cortex        | Prefrontal   |
| VIspm        | posteromedial visual area             | Medial       |
| Ald          | Agranular insular area, dorsal part   | Lateral      |
| Alv          | Agranular insular area, ventral part | Lateral      |
| Alp          | Agranular insular area, posterior part | Lateral    |
| GU           | Gustatory areas                      | Lateral      |
| VISc         | Visceral area                         | Lateral      |
| TEa          | Temporal association areas            | Lateral      |
| PERI         | Perirhinal cortex                    | Lateral      |
| ECT          | Ectorhinal area                       | Lateral      |
| VI-sal       | Anterolateral visual area             | Visual       |
| VI-si        | Lateral visual area                  | Visual       |
| VI-sp        | Primary visual area                  | Visual       |
| VI-spl       | Posterolateral visual area            | Visual       |
| VI-sii       | Laterointermediate area               | Visual       |
| VI-spor      | Postrhinal area                       | Visual       |
| VI-srl       | Rostrolateral visual area             | Visual       |
| AUDd         | Dorsal auditory area                  | Auditory     |
| AUDp         | Primary auditory area                 | Auditory     |
| AUDpo        | Posterior auditory area               | Auditory     |
| AUDv         | Ventral auditory area                 | Auditory     |
| HPF          | Hippocampal Formation                 |              |
| CA1          | Field CA1                             |              |
| CA2          | Field CA2                             |              |
| CA3          | Field CA3                             |              |
| DG           | Dentate gyrus                         |              |
| ENTI         | Entorhinal area, lateral part         |              |
| ENm          | Entorhinal area, medial part          |              |
| CTXsp        | Cortical Subplate                     |              |
| CLA          | Claustrum                             |              |
| OLF          | Olfactory Areas                       |              |
| AOB          | Accessory olfactory bulb              |              |
| AON          | Anterior olfactory nucleus            |              |
| TT           | Taenia tecta                          |              |
| Abbreviation | Description |
|--------------|-------------|
| DP           | Dorsal peduncular area |
| STR          | Striatum     |
| CP           | Caudoputamen  |
| ACB          | Nucleus accumbens |
| LSc          | Lateral septal nucleus, caudal (caudodorsal) part |
| TH           | Thalamus     |
| LP           | Lateral posterior nucleus of the thalamus |
| PO           | Posterior complex of the thalamus |
| IAM          | Interanteromedial nucleus of the thalamus |
| IAD          | Interanterodorsal nucleus of the thalamus |
| IMD          | Intermediodorsal nucleus of the thalamus |
| MD           | Mediodorsal nucleus of the thalamus |
| RH           | Rhomboid nucleus |
| CM           | Central medial nucleus of the thalamus |
| PCN          | Paracentral nucleus |
| CL           | Central lateral nucleus of the thalamus |
| PF           | Parafascicular nucleus |
| MB           | Midbrain     |
| SCs          | Superior Colliculus, sensory-related |
| PAL          | Pallidum     |

Table S1 related to Figure 1 and all text. Abbreviations.
| Structure    | % of Structure in core-DMN Mask (z>1.7) | % of Structure in DMN Mask (z>1) | Fraction of projections in DMN (mean) | Fraction of projections in DMN (SD) | DMN Coefficient (mean) | DMN Coefficient (SD) | Number of Experiments |
|--------------|----------------------------------------|----------------------------------|--------------------------------------|-------------------------------------|------------------------|----------------------|-----------------------|
| ACAv         | 100                                    | 100                              | 0.93                                 | 0.03                                | 0.22                   | 0.1                  | 5                     |
| ACAd         | 99                                     | 99                               | 0.9                                  | 0.04                                | 0.2                    | 0.1                  | 11                    |
| SSp-tr       | 98                                     | 99                               | 0.81                                 | 0.02                                | 0.06                   | 0.05                 | 2                     |
| ILA          | 97                                     | 99                               | 0.89                                 | 0.05                                | 0.11                   | 0                    | 2                     |
| SSp-II       | 97                                     | 98                               | 0.72                                 | 0.06                                | 0.01                   | 0.04                 | 6                     |
| PL           | 89                                     | 89                               | 0.81                                 | 0.04                                | 0.12                   | 0.09                 | 3                     |
| ORBv         | 89                                     | 93                               | 0.79                                 | 0.03                                | 0.25                   | 0.1                  | 5                     |
| VI Sa        | 87                                     | 96                               | 0.77                                 | 0.03                                | 0.08                   | 0.02                 | 2                     |
| ORBm         | 84                                     | 88                               | 0.78                                 | 0.03                                | 0.19                   | 0.06                 | 3                     |
| ORBI         | 77                                     | 93                               | 0.67                                 | 0.12                                | 0.05                   | 0.15                 | 5                     |
| VI Sam       | 72                                     | 85                               | 0.66                                 | 0.06                                | 0.04                   | 0.03                 | 8                     |
| RSPd         | 64                                     | 70                               | 0.45                                 | 0.3                                 | -0.04                  | 0.03                 | 6                     |
| RSPv         | 67                                     | 78                               | 0.69                                 | 0.19                                | 0                      | 0.04                 | 11                    |
| MoS          | 52                                     | 64                               | 0.46                                 | 0.29                                | -0.19                  | 0.29                 | 18                    |
| RSPag        | 51                                     | 62                               | 0.6                                  | 0.22                                | 0.02                   | 0.05                 | 8                     |
| V Srl        | 42                                     | 62                               | 0.64                                 | 0.04                                | 0.09                   | 0.02                 | 6                     |
| VI Sm        | 32                                     | 63                               | 0.51                                 | 0.09                                | -0.01                  | 0.04                 | 8                     |
| Ald          | 10                                     | 61                               | 0.4                                  | 0.15                                | -0.11                  | 0.09                 | 3                     |
| Alv          | 5                                      | 53                               | 0.56                                 | 0                                  | 0.03                   | 0                    | 1                     |
| SSp-ul       | 30                                     | 39                               | 0.56                                 | 0.29                                | -0.07                  | 0.07                 | 5                     |
| MO p         | 28                                     | 40                               | 0.43                                 | 0.27                                | -0.19                  | 0.23                 | 9                     |
| SSp-un       | 24                                     | 32                               | 0.48                                 | 0                                  | -0.01                  | 0                    | 1                     |
| FRP          | 20                                     | 30                               | 0.37                                 | 0                                  | -0.17                  | 0                    | 1                     |
| SSp-bfd      | 16                                     | 27                               | 0.5                                  | 0.18                                | 0                      | 0.05                 | 13                    |
| VI Sp        | 6                                      | 12                               | 0.23                                 | 0.17                                | -0.04                  | 0.04                 | 80                    |
| SS s         | 0                                      | 0                                | 0.13                                 | 0.09                                | -0.24                  | 0.14                 | 7                     |
| SSp-n        | 0                                      | 0                                | 0.09                                 | 0.03                                | -0.23                  | 0.07                 | 5                     |
| SSp-m        | 0                                      | 0                                | 0.07                                 | 0.05                                | -0.27                  | 0.15                 | 7                     |
| Aip          | 0                                      | 0                                | 0.16                                 | 0                                  | -0.09                  | 0                    | 1                     |
| GU           | 0                                      | 1                                | 0.08                                 | 0.08                                | -0.09                  | 0.11                 | 2                     |
| V ISC        | 0                                      | 0                                | 0.19                                 | 0.07                                | -0.26                  | 0.12                 | 4                     |
| Tea          | 0                                      | 1                                | N/A                                  | N/A                                 | N/A                    | N/A                  | 0                     |
| PERI         | 0                                      | 0                                | N/A                                  | N/A                                 | N/A                    | N/A                  | 0                     |
| ECT          | 0                                      | 0                                | 0.16                                 | 0.15                                | -0.04                  | 0                    | 2                     |
| VI Sal       | 0                                      | 23                               | 0.38                                 | 0.04                                | 0                      | 0.01                 | 5                     |
| VI SI        | 0                                      | 0                                | 0.21                                 | 0.06                                | -0.05                  | 0.03                 | 12                    |
| VI Spl       | 0                                      | 1                                | 0.06                                 | 0.02                                | -0.06                  | 0.07                 | 3                     |
| VI Si        | 0                                      | 3                                | 0.17                                 | 0.05                                | -0.08                  | 0.02                 | 2                     |
| VI Spor      | 0                                      | 0                                | 0.12                                 | 0.04                                | -0.09                  | 0.02                 | 11                    |
| AUDd         | 0                                      | 21                               | 0.34                                 | 0.03                                | -0.04                  | 0.04                 | 2                     |
| AUDp         | 0                                      | 12                               | 0.23                                 | 0.08                                | -0.06                  | 0.02                 | 7                     |
| AUD po       | 0                                      | 26                               | 0.28                                 | 0.13                                | -0.03                  | 0.02                 | 7                     |
| AUDv         | 0                                      | 0                                | 0.12                                 | -0.1                                | 0                      | 1                    | 1                     |

Table S3 related to Figure 1. Cortical structures designated as in-DMN, out-DMN, and borderline for DMN membership (assigned to out-DMN). The fraction of each structure in the DMN and core masks, mean and standard deviation (SD) for the fraction of projections in the DMN for WT injection experiments in each structure, mean and SD for the DMN coefficient for WT injection experiments in each structure, and number of WT injections per structure are provided. Abbreviations in Table S1.