1 Title Page

2 **Title:** A weighted graph of the projections to mouse auditory cortex

3 **Running title:** Projections to mouse auditory cortex

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21 **Abstract**

22 The projections to individual cortical areas from extrinsic sources are a major determinant
 23 of the area's function, but we lack comprehensive quantitative input maps even for primary
 24 sensory areas in most model species. To quantify all input sources to the mouse primary
 25 auditory cortex (Au1), we made localized injections of modified rabies virus (SADΔG-
 26 mCherry) into Au1 of five C57BL/6 mice and identified all the cortical and subcortical areas
 27 containing retrogradely labeled cells. Of all neurons projecting to Au1 from extrinsic areas,
 28 27 % were located in the ipsilateral cortex, 14 % in the contralateral cortex, and 58 % in
 29 subcortical regions (almost exclusively ipsilateral, predominantly in the medial geniculate
 30 nucleus). Although 90 % of the labeled cells in the ipsilateral cortex were located within
 31 1 mm of Au1, most cortical areas projected to Au1, including visual, somatosensory, motor,
 32 rhinal, cingulate and piriform cortices. The hierarchical relations of the cortical areas
 33 projecting to Au1 were determined based on the proportion of cell bodies in superficial
 34 versus deep layers. Feedback projections (from deep layers 5/6) dominated, but temporal
 35 association and auditory cortices were on the same hierarchical level, providing input from
 36 both superficial and deep layers. Au1 is embedded in a densely connected network that
 37 involves a high degree of cross-modal integration.

38 Introduction

39 New insights into the rules of inter-areal connectivity of neocortical circuits have been
 40 given by quantitative studies of the macaque monkey where retrograde tracing techniques
 41 combined with quantitative data collection and sophisticated statistical and graph theoretic
 42 analyses have completely revolutionized our concepts of cortical wiring in a primate brain
 43 (Markov et al., 2011, 2012, 2013; Ercsey-Ravasz et al., 2013). Whereas before macaque
 44 cortex was seen as a modular organization of sensory, motor and association areas linked
 45 in a 'small world' network, it is now clear that the networks are not small world, but dense,
 46 and the probability of connection between areas declines exponentially with physical
 47 distance. This latter feature means that the connections between nearest neighboring
 48 areas approach all-to-all, but long-range connections form a very small fraction of the total
 49 complement of input to an area.

50

51 In the comparatively tiny brain of the mouse, such quantitative retrograde studies have not
 52 been attempted, so it remains to be discovered whether the same rules of connectivity
 53 apply. One reason for the lack of comprehensive, detailed and quantitative studies is
 54 technical: cortical areas in the macaque are large, so large pressure injections of
 55 conventional tracers can be placed with high precision. But in the mouse, even primary
 56 areas are small, e.g. area 41 (primary auditory cortex, Au1) is only 0.8 mm² in area, and
 57 thus injecting sufficient amounts of conventional retrograde tracers with high accuracy is a
 58 challenge. Work on mouse cortical connectivity has revealed details of inter-areal
 59 projection maps of a number of visual areas (Wang and Burkhalter, 2007), and now a
 60 major goal is to describe the mouse 'connectome' by performing large-scale tracing
 61 studies (Oh et al., 2014; Zingg et al., 2014). These studies have not, however, exploited
 62 the quantitative methods used so successfully in the macaque.

63

64 In this study of the inputs to the primary auditory cortex in the mouse we adopted the
65 methods pioneered in the macaque studies. The prime advantage of using retrograde
66 rather than anterograde tracing methods is that counting neurons is far simpler than
67 counting synapses. The number of retrogradely labeled neurons makes for a clearly
68 defined measure of relative input strengths of different source areas to a target area. The
69 cortical laminae where the projecting neurons are located provides additional information
70 about the nature of the connection to the target area and gives hints concerning the
71 relative hierarchical position of cortical source and target area (Rockland and Pandya,
72 1979; Barone et al., 2000). Thus here we have expressed the relative connection 'weight'
73 of each area as the fraction of retrogradely labeled neurons (FLN, Markov et al., 2011) and
74 further determined the fraction of labeled supragranular layer neurons (SLN, Barone et al.,
75 2000) as a measure of 'feedforward' versus 'feedback' projections for cortical areas.

76

77 These data answer the important question of whether the principles of interareal
78 connectivity discovered in the macaque also apply to mouse neocortex. Unlike in other
79 mouse connectivity studies, the distribution of FLN and SLN values we found can be
80 directly compared to the data from macaques. At a time in which the mouse is the most
81 widely used model system in neuroscience, it is becoming more relevant to link
82 quantitative findings on rodent brain networks and function to the extensive work in
83 primates.

84 **Materials and Methods**

85 All experiments were performed in agreement with European Communities Council
86 Directive of November 24, 1986 (86/609/EEC), the Veterinary Office of Zurich guidelines
87 under Licence No. 16/2010 to K.A.C. Martin and in accordance with the Basel Declaration
88 principles. Seven young adult male C57BL/6 mice (B6CBAF1/OlaHsd (C57BL/6 x CBA),
89 Harlan lab, Netherlands) were used for this study, five for virus injections and two for BDA
90 injections. The adeno-associated virus AAV2/5.CMV.EGFP encoding enhanced green
91 fluorescent protein (EGFP) was purchased from Penn Vector Core, Pennsylvania. The
92 glycoprotein-deleted rabies virus encoding the red fluorophore mCherry [SAD Δ G-mCherry]
93 (Marshall et al., 2010) was a generous gift from Botond Roska, Friedrich Miescher Institute
94 for Biomedical Research, Basel, Switzerland (Yonehara et al., 2011).

95 **Surgical procedures and perfusion**

96 After induction of anaesthesia with 4 % isoflurane in 100 % oxygen, the mouse was fixed
97 through a lidocaine-covered mouthpiece and prepared for stereotaxic surgery. During the
98 surgery, the isoflurane level was reduced to 2-3 % and the body temperature of the mouse
99 was monitored and maintained at 37 °C. From the Paxinos and Franklin atlas (Paxinos
100 and Franklin, 2001), the coordinates for the left primary auditory cortex (Au1), were chosen
101 to be 2.9 mm posterior and 4.1 mm lateral in relation to Bregma and the injection pipette
102 was positioned at an angle of 30 degrees from the horizontal axis. A distinct Y-shaped
103 blood vessel immediately posterior to Au1 served as an additional reference point for the
104 injection site. The location of injection was later confirmed in histological sections.

105

106 Mice were injected with rabies virus or a mixture of adeno-associated virus (AAV, 1/4 to 1/3
107 of the virus mixture) and rabies virus (3/4 to 2/3 of the virus mixture) or with biotinylated

108 dextran amine (BDA, MW 10000, Invitrogen) through a micro-pipette of 10-20 μ m inner tip
 109 diameter. AAV was used to identify the maximum extent of the injection site, and rabies as
 110 the tracer, which retrogradely labels neurons with terminals in the injection site. The exact
 111 titer of the rabies virus was unknown; but it is expected to have been up to 10^{10} infectious
 112 units per ml (Wickersham et al., 2010). A volume of 100-200 nl of the virus mixture was
 113 injected via a glass pipette by pressure. In mice 1 and 2 the injection site was centered in
 114 layer 4 and 5, but all layers (except the deepest part of layer 6) were labeled. In the other
 115 mice small volumes were injected ca. every 200 μ m to label more evenly the whole depth
 116 of Au1. A second set of mice were injected iontophoretically with BDA-MW10000 dissolved
 117 1:50 in electrophoretic solution (0.2 M KCl, 0.05 M Tris Base, pH 7.9) instead of with virus.
 118 We started injecting (2-3 μ A; 5 s on, 5 s off) at ca. 800 μ m depth and moved up 100 μ m
 119 every 2 min, so the total injection time was about 20 min. Post-injection, the mice were
 120 kept for 7 to 10 days to allow for the spread of the viruses and the expression of the
 121 fluorescent proteins. BDA-injected mice recovered for 2 weeks.

122

123 The mice injected with rabies were then transcardially perfused under deep anesthesia
 124 (100 mg/kg sodium pentobarbital administered intraperitoneally). After 0.1 ml heparin
 125 (10 % in Ringer solution) was injected into the left ventricle of the heart to prevent blood
 126 clotting, the brain was perfused with saline (0.9 % NaCl), followed by 4 % paraformaldehyde
 127 (PFA, 200-300 ml in total). The brain was removed and kept in 0.1 M phosphate buffer (pH
 128 7.4) overnight or in 4 % PFA until further processing. The BDA-injected mice were
 129 perfused with 4 % PFA, 15 % picric acid and (freshly added) 0.8 % glutaraldehyde,
 130 followed by a sucrose-gradient (10 %, 20 % and 30 % sucrose) perfusion. Then the brains
 131 were sunk in 30 % sucrose.

132 Preparation for microscopy

133 The fixed brains were embedded in 2 % agarose. For the fluorescence microscopy, the
134 coronal sections of 60 μm thickness were cut with a Microm HM 650 V vibratome. BDA+
135 brains were freeze-thawed (i.e. held in a plastic beaker which was dipped in liquid nitrogen
136 until the brain was completely white and then transferred quickly to 0.1 M phosphate
137 buffer) and coronal sections were cut at 80 μm . The 60 μm sections were stained for NeuN
138 (neuronal nuclei), which also labels neuronal cell bodies (Mullen et al., 1992), in order to
139 determine the cortical layers and areal borders. The following protocol was used: Blocking
140 and permeabilisation in 2 % PB-Tx (2 % TritonX-100 in 0.1 M phosphate buffer) with 10 %
141 horse serum for 2 h at room temperature, followed by incubating the sections in the
142 primary antibody mouse- α -NeuN (Millipore, MAB377, AB_2298772) diluted 1:500 at 4 °C
143 for 2 days. The sections were then incubated with the secondary antibody donkey- α -
144 mouse-AlexaFluor488 (Invitrogen, A-21202, AB_2535788, mice 1 and 3 to 5) or donkey- α -
145 mouse-AMCA (aminomethyl-coumarin acetate, Jackson, 715-155-151, AB_2340807,
146 mouse 2), diluted 1:500 for 6 h or overnight at 4 °C. Between antibody incubations, the
147 sections were washed with 0.4 % PB-Tx, after the incubation with secondary antibody, the
148 washes were done with PB only.

149

150 The BDA sections were washed in TB pH 8 (Tris buffer, 6.06 g Trizma.HCl and 1.36 g
151 Trizma.Base in 1 l ddH₂O). To visualize the BDA, the ABC kit (Avidin and Biotinylated
152 horseradish peroxidase macromolecular Complex kit, Vector Lab) was applied. The
153 sections were then incubated in 0.6 % $(\text{NH}_4)_2\text{Ni}(\text{SO}_4)_2 \cdot 6 \text{H}_2\text{O}$ (ammonium Ni(II) sulfate
154 hexahydrate) and 0.015 % DAB (3,3'-Diaminobenzidine) in TB pH 8 for 20 min. The
155 catalyst H_2O_2 was added (to a final concentration of 0.005 %) and the reaction stopped
156 after 3-5 min with the last washes in PB. The sections were mounted with DAPI (4',6-

157 diamidino-2-phenylindole, Vectashield mounting medium, H-1200, AB_2336790) onto
158 slides for microscopy. (The AMCA-stained sections were mounted without DAPI because
159 of the overlap in the emission frequencies of both fluorophores).

160

161 The BDA sections were mounted on gelatine-coated slides, air-dried and stained with
162 Neutral red or Cresyl violet. For the staining, the sections were dehydrated and rehydrated
163 in ascending and descending ethanol series. The tissue was incubated in Neutral red or
164 Cresyl violet for ca. 7 min. Then followed a second dehydration series involving a
165 differentiation step in 95 % ethanol acidified with acetic acid (ca. 1 %). After additional
166 15 min in 100 % ethanol, the sections were transferred to xylene and finally mounted with
167 DPX. Overview micrographs of the sections were taken at 1.25x magnification and
168 micrographs for counting and attribution to cortical layers and areas were taken at 10x
169 magnification with a Leica DMRB fluorescence or light microscope and at 20x with a Zeiss
170 Mirax Midi Slide Scanner.

171 **Analysis**

172 The BDA-labeled brains were used for a qualitative comparison with the modified rabies
173 virus label. The labeling pattern was very similar between the two tracers, but the BDA
174 labeling was far sparser and only animals labeled with the more sensitive modified rabies
175 tracer were analyzed quantitatively. The labeled cells were counted manually under the
176 microscope and assigned to the corresponding areas based on the 10x and 20x
177 micrographs. The identification of different cortical areas was based on detailed
178 cytoarchitectonic characterization using the description of Caviness (1975) and the mouse
179 brain atlas of Paxinos and Franklin (2001). The first step was to mark the boundaries of
180 cortical layers based on cell density and soma size using the NeuN labeling. We used
181 NeuN staining in addition to DAPI since it provides a clearer definition of layer and area

182 borders, comparable to the Nissl staining. Boundaries were marked for every area of every
183 section where a labeled cell was found, as well as in adjacent sections in order to confirm
184 consistency of layer location. When cells were found exactly on layer boundaries, they
185 were assigned to the lower layer in even sections and to the upper layer in odd sections.
186 The boundaries between all cortical areas were then manually identified for each section in
187 each animal by careful examination of the changes in cell densities and cortical layer
188 thickness (as in Caviness, 1975).

189

190 Subcortical cells were mostly aligned to the mouse atlas, as the area boundaries could not
191 always be determined cyto-architectonically. While functional or tracing methods offer the
192 possibility of an even finer subdivision of cortical areas (e.g. Stiebler et al., 1997; Tsukano
193 et al., 2015, for auditory cortex; and Wang and Burkhalter, 2007; Garrett et al., 2014, for
194 visual cortex), they cannot offer the comprehensive assignment of every cell to a known
195 location that can only be achieved with the classical cytoarchitectonic method.

196

197 Images taken with the Zeiss Mirax Midi Slide Scanner were exported with program
198 PannoramicViewer. The different channels of the fluorescence micrographs were overlaid
199 and brightness and contrast adjusted using GIMP (GNU image manipulation program,
200 <http://www.gimp.org>, RRID: SCR_003182) or Adobe Photoshop CS6 ([https://www.adobe](https://www.adobe.com/products/photoshop.html)
201 [.com/products/photoshop.html](https://www.adobe.com/products/photoshop.html), RRID: SCR_014199). Using the Fiji plug-in TrakEM2 (Fiji,
202 <http://fiji.sc>, RRID: SCR_002285, TrakEM2, [https://www.ini.uzh.ch/~acardona/trakem2](https://www.ini.uzh.ch/~acardona/trakem2.html)
203 [.html](https://www.ini.uzh.ch/~acardona/trakem2.html), RRID: SCR_008954, Cardona et al., 2012), the micrographs of the labeled cells
204 were manually aligned to the coronal sections of the mouse atlas and the volumes of the
205 injection sites (i.e. the spread of the AAV) was measured. The number of cells per area
206 and cortical layer was listed in a LibreOffice table, which was converted into a csv file. The

207 data were then analyzed and graphed with Matlab (Mathworks, [https://ch.mathworks.com/](https://ch.mathworks.com/products/matlab.html)
 208 products/matlab.html, RRID: SCR_001622) or depicted with Adobe Illustrator CS6
 209 (<https://www.adobe.com/products/illustrator.html>, RRID: SCR_014198) and Photoshop,
 210 respectively.

211

212 The fraction of labeled neurons (FLN, Markov et al., 2011) equals the fraction of labeled
 213 cells in a source area divided by the total number of labeled cells in the whole brain in
 214 percent. The extrinsic fraction of labeled neurons (FLNe, Markov et al., 2011) is the
 215 fraction of labeled cells in a source area divided by the total number of labeled cells
 216 excluding the injection site areas Au1 and AuV of the left hemisphere. For cortical areas,
 217 the fraction of labeled supragranular layer neurons (SLN, Barone et al., 2000) was
 218 calculated by dividing the number of neurons in layers 1-4 over the number of all labeled
 219 neurons in this area.

220

$$221 \quad FLN = \frac{\text{number of labeled cells in a source area}}{\text{total number of labeled cells in the brain}} \cdot 100 \%$$

222

$$223 \quad FLNe = \frac{\text{number of labeled cells in a source area}}{\text{total number of labeled cells in the brain except in left Au1 and AuV}} \cdot 100 \%$$

224

$$225 \quad SLN = \frac{\text{number of labeled cells in the supragranular layers of a source area}}{\text{total number of labeled cells in the source area}}$$

226 Results

227 To obtain the complete input map of neurons connecting to Au1 from both hemispheres,
 228 single pressure injections of modified rabies virus together with AAV were made in Au1 of
 229 the left hemisphere in five mice. The location of the injection was confirmed in histological
 230 sections labeled with NeuN using cytoarchitectonical features to identify Au1. The
 231 injections sites were clearly visible under the epifluorescence microscope and confirmed
 232 that the modified rabies was injected through all layers of Au1 (see Figure 1A+B). The
 233 injection site volumes and locations were comparable between animals. The average
 234 injection site volume was $0.70 \pm 0.19 \text{ mm}^3$ and the virus reached a depth of approximately
 235 0.9 mm (cortical depth of Au1 was 1 mm). At the center of the injection site, the distance
 236 from the rhinal fissure along the cortical surface to the center of the injection site was
 237 $1.44 \pm 0.05 \text{ mm}$, which corresponded to a very consistent $20.3 \pm 0.2 \%$ of the total
 238 distance from rhinal fissure to the midline in each brain. Examples of coronal sections
 239 containing labeled neurons (in magenta) and stained for NeuN (in green) for cyto-
 240 architectonic identification of cortical areas are shown in Figure 2A. Note the relatively
 241 dense labeling in the ipsi- and contralateral auditory cortices and in the medial geniculate
 242 nucleus of the thalamus, as well as distributed label in association cortical areas, and
 243 rhinal, visual, somatosensory and motor cortex.

244

245 Figure 1A+B shows an injection site of the viruses nine days after the injection. We co-
 246 injected AAV because after the long survival times we found that the rabies was far less
 247 visible at the injection site, which made the limits of the rabies injection difficult to
 248 demarcate. The extent of the rabies was confined to Au1, but the AAV filled most of Au1
 249 and a portion of the secondary auditory cortex, AuV, which lies more ventrally. It was thus
 250 possible that some of the rabies particles had also reached the AuV and therefore we were

conservative and assumed that the injection site included both Au1 and AuV. Thus when referring to projections to Au1, we implicitly include AuV. In one animal the tip of the pipette touched the hippocampus immediately below Au1, and the modified rabies labeled hippocampal neurons and possibly also a few of the projections to area CA1. This animal was excluded from the final quantitative analysis, although the pattern and weights of inputs to Au1 – after taking into account the intra-hippocampal connections – was similar to the brains where the injection was confined to the neocortex. In another animal, the injection labeled a small total number of neurons (ca. 1000). The major input sources of Au1 were the same as in the larger injections, but the numbers were too few to provide accurate counts and this was excluded from the quantitative analysis.

Fraction of labeled neurons in extrinsic sources (FLNe)

The size of the pressure injection site was relatively large and covered more than half the volume of Au1, so although the fraction of labeled cells in Au1 was $26.5 \pm 1.1\%$ of all labeled cells, this is likely to be an underestimate since we excluded cells within or at the border of the injection site that might have taken up the virus directly through their soma or dendrites. Thus to calculate the weights of the projections, we excluded the labeled neuron in Au1 and AuV and expressed the counts as a 'fraction of labeled neurons extrinsic' (FLNe), as a proportion of total labeled cells in the surrounding brain areas (Markov et al., 2011). The FLNe provides a simple measure of the numerical 'weight' of the many projections to Au1/AuV. The averaged FLNe values for the three mice were ranked by magnitude and plotted on a log axis (Figure 1C).

Thirty-four of the 47 neocortical areas identified contained neurons projecting to Au1. These areas were identified based on their cytoarchitectonic properties in NeuN-stained sections (see Methods for details). The most prominent labeling occurred in the auditory

276 cortices (Figure 1C). The ipsilateral AuD (secondary auditory cortex, dorsal part) was the
 277 most densely labeled cortical area with an FLNe of 12.7 ± 5.7 % (i.e. mean $\pm$ standard
 278 deviation). The ipsilateral temporal association cortex (TeA, FLNe 7.5 ± 4.2 %) provided
 279 major projections to Au1/AuV. Although TeA is the association cortex that is physically
 280 closest to the auditory cortex, it was not the only association area to provide input to
 281 Au1/AuV, since the parietal association cortex (LPtA, MPtA, FLNe 0.08 ± 0.12 %) and
 282 even the distant frontal association cortex (FrA, FLNe 0.04 ± 0.06 %) projected to Au1.

283

284 A striking finding was the high degree of direct cross-modal projections to Au1, both in
 285 terms of projection weight and number of areas involved. The visual cortices were highly
 286 connected with Au1/AuV, having a cumulative FLNe of 3.3 ± 1.5 %. The lateral secondary
 287 visual cortex (V2L) contributed the biggest part, while the primary visual cortex (V1) and
 288 the medial secondary visual cortices (V2MM, V2ML) contributed less than 1 % each. Other
 289 sensory modalities formed weaker projections. The somatosensory cortex (primary and
 290 secondary) had a cumulative FLNe of 1.2 ± 1.0 %. Although the secondary somatosensory
 291 cortex (S2) provided a stronger projection than the primary somatosensory cortex (S1),
 292 labeled neurons were spread across the whole somatotopic representation in the primary
 293 somatosensory cortex (S1, S1BF, S1HL, S1Sh and S1Tr), with the barrel field (S1BF)
 294 being the strongest of these.

295

296 The olfactory system was also linked to the auditory cortex via a direct projection from the
 297 olfactory association cortex or piriform cortex (Pir, FLNe 0.09 ± 0.06 %), which is part of
 298 the three-layered archicortex. Weak projections also originated from the gustatory or
 299 insular cortex. Both granular and agranular parts of the insular cortex contained labeled
 300 neurons, but none of these regions were labeled in all animals, which is reflected in the

301 low FLNe values (GI, DI and AIP, cumulative FLNe 0.11 ± 0.10 %).

302

303 The Au1/AuV not only received input from sensory cortical areas, but also from the primary
304 and secondary motor cortex (M1, M2, FLNe 0.39 ± 0.08 %). These projections did not
305 involve large numbers of neurons, but they were seen consistently in all brains. Moreover,
306 the rhinal cortices of the parahippocampal region were the source of a substantial input to
307 Au1. These regions included the ectorhinal cortex (Ect), lying on the ventral border of the
308 temporal association cortex (TeA), and successively the areas of perirhinal (PRh) and
309 lateral (LEnt) and medial (MEnt) entorhinal cortex. Altogether, the FLNe of the rhinal cortex
310 was 1.7 ± 0.8 %. Sparser projections originated in the medial cortices: the cingulate and
311 retrosplenial cortex had a cumulative FLNe of 0.32 ± 0.42 %. The labeling in the
312 retrosplenial cortex (RSA and RSG, also called the posterior cingulate cortex), was denser
313 than the labeling in the transition zone Cg/RS and in the cingulate cortex (or anterior
314 cingulate cortex, Cg1 and Cg2). Also associated with the limbic system, the orbital cortex
315 sent a weak connection to the auditory cortex. Lateral (LO), ventral (VO) and dorsolateral
316 (DLO) orbital cortex contributed 0.05 ± 0.09 % to the total FLNe.

317

318 Most of the same cortical areas labeled in the ipsilateral cortex were also labeled in the
319 contralateral cortex, but with a much smaller weight. The total FLNe of the contralateral
320 cortex was 14.3 ± 3.4 %, whereas the FLNe of the ipsilateral cortex 27.4 ± 6.0 %. Note
321 that the ipsilateral FLNe is an underestimate because the local intra-areal input around the
322 injection site could not be included in this calculation. For single areas, the projection from
323 the corresponding area in the contralateral cortex was in most cases much weaker. This
324 difference in input weight between hemispheres was especially evident in areas with a
325 relatively high ipsilateral FLNe value; the contralateral AuD, for instance, provided a ten-

fold weaker projection than the ipsilateral AuD. Eighteen cortical areas were labeled in the contralateral hemisphere and involved areas of the auditory, visual and somatosensory modality, motor and association cortex as well as rhinal cortex (Figure 1C; Figure 2B). Subcortical labeling, however, was very sparse in the contralateral hemisphere. Only three nuclei were labeled with 1 or 2 cells each, whereas on the ipsilateral side, labeled neurons were found in 63 different subcortical nuclei. These were mostly concentrated in the auditory thalamus, but spread as far as the cortical subplate and the deep midbrain. Overall, the FLNe from ipsilateral subcortical areas was 58.3 ± 3.3 %. Relatively strong projections were found from the basal ganglia, including the striatum (caudate/putamen, CPu, FLNe 1.44 ± 0.65 %), globus pallidus (LGP, MGP, FLNe 3.6 ± 1.1 %), substantia nigra and subthalamic nucleus. Of these areas, the lateral globus pallidus (LGP) had the most labeled cells, followed by CPu.

338

In the mice we injected with BDA, we also found widespread anterogradely labeled fibers in the caudate putamen and the lateral globus pallidus. In the mouse with a bigger BDA injection, there were also a few retrogradely labeled cell bodies inside these areas, but mostly close to the surrounding brain areas. Also some of the rabies-positive cells in the caudate putamen and the lateral globus pallidus were close the transition zones to the amygdala or the basal forebrain, but in addition many of the rabies-positive cells were clearly inside the striatum and pallidum. In contrast to the BDA-labeled axons, even the rabies-positive cells were restricted to the dorsal part of the pallidum and striatum, thus calling the connection between caudate putamen / globus pallidus and Au1 'reciprocal' may not be strictly correct.

349

Other areas linked to the basal ganglia had a few labeled cells, namely the zona incerta

351 (ZI) and the reticular thalamic nucleus (Rt). The zona incerta splits into a dorsal (ZID) and
 352 a ventral (ZIV) part, which are easily distinguishable as two parallel beams of cells in a
 353 coronal section. Together with the subincertal nucleus (SubI), the FLNe of the zona incerta
 354 was 0.18 ± 0.22 %.

355

356 Several nuclei of the basal forebrain projected to Au1, with a cumulative FLNe of
 357 0.3 ± 0.1 %. They included the substantia innominata (SI), the basal nucleus (B) and the
 358 horizontal limb of the diagonal band (HDB). Of these areas, the substantia innominata was
 359 consistently labeled in all animals. Labeled areas in the cortical subplate involved the
 360 endopiriform cortex (VEn) and the claustrum (CI), which projected to Au1/AuV from both
 361 hemispheres. With a cumulative FLNe of 0.08 ± 0.04 %, these projections were of a low
 362 weight.

363

364 Although the thalamus is expected to be one of the main input sources of a primary
 365 cortical area such as Au1, the projections from the thalamus were surprisingly numerous
 366 and together constituted close to half of the total extrinsic projection weights, i.e.
 367 48.6 ± 3.8 % of the FLNe. The main contributor was the ventral medial geniculate nucleus
 368 (MGV – the auditory thalamus) with an FLNe of 35.5 ± 6.7 %. Surrounding this dense
 369 cluster of retrogradely labeled neurons, the other nuclei of the medial geniculate body
 370 (MGD, MGM, MZMG, SG) contributed 11.2 ± 4.4 % to the FLNe. The labeling in the
 371 thalamus extended beyond the medial geniculate body to various nuclei of the ventral,
 372 lateral and posterior thalamus. These thalamic nuclei typically had a projection weight of
 373 less than 0.3 %. In total, including the five areas of the medial geniculate body, 26 thalamic
 374 nuclei were labeled.

375

376 The midbrain also provided a number of projections. The most prominent of these was
 377 from the inferior colliculus (BIC) including the subjacent subbrachial nucleus (SubB),
 378 providing a total FLNe of 1.8 ± 0.7 %. A number of neurons projecting to Au1 were located
 379 in the deep mesenchymal nucleus (DpMe, FLNe 0.9 ± 1.3 %). The Au1 received direct
 380 input from nuclei known to modulate cortical activity such as the anterior pretectum and
 381 the dorsal raphe nucleus, but both areas provided a low input weight: The FLNe of the
 382 anterior pretectum (APTD, APTV, APT) amounted to only 0.15 ± 0.16 % and the dorsal
 383 raphe nucleus (DRC) had an FLNe of 0.01 ± 0.02 %. The substantia nigra, also a midbrain
 384 nucleus, sent projections from both the lateral (SNL) and the reticular (SNR) part with an
 385 FLNe of 0.06 ± 0.08 %.

386
 387 A small number of neurons were consistently labeled in the hippocampus (Or, Py, FLNe
 388 0.5 ± 0.4 %) and the subiculum (S, PrS, FLNe 0.14 ± 0.17 %). In the hippocampus the
 389 labeled neurons were mainly in the CA1 region close to Au1 in the oriens and pyramidal
 390 cell layers. These lay some distance from the injection site, indicating they were labeled
 391 retrogradely. The labeled hypothalamic cells were distributed among four nuclei, of which
 392 the lateral hypothalamic nucleus (LH) contained most cells. The cumulative FLNe of the
 393 labeled hypothalamic nuclei (LH, DM, Te, SuMM) was 0.14 ± 0.11 %. Finally, the amygdala
 394 complex was found to have a direct connection to Au1: from the anterior (AAD, AAV) and
 395 basomedial (BMP) amygdaloid nuclei as well as from the amygdalostriatal transition area
 396 (AStr). The FLNe of the amygdala was 0.08 ± 0.03 %, indicating a weak projection.

397
 398 Across the whole brain, the FLNe spanned four orders of magnitude (Figure 1C). Areas
 399 with high FLNe values were consistently labeled in all mice. This was typically the case for
 400 FLNe values above 0.18 %, with two exceptions (DpMe and ic: internal capsule). Low

401 FLNe values did not necessarily mean that the projections were variable, since several
 402 sources with an FLNe below 1 % were found in all mice, most notably the projection from
 403 the ipsilateral claustrum (FLNe 0.06 %). The strongest connection that was found only in
 404 one animal (from PLi, posterior limitans thalamic nucleus: FLNe 0.08 %) had a similar
 405 weight.

406

407 Of all the 98 areas with an observed projection to Au1, 30 were labeled in all animals and
 408 had a cumulative FLNe projection weight of approximately 96 %. Thirty-two areas were
 409 only found labeled in one animal. Most of these projections originated in subcortical areas
 410 and they typically contained only 1-2 cells per area. The cumulative FLNe of these
 411 uniquely labeled areas was less than 1 %.

412

413 Distance effects were clearly in evidence: the closer a cortical area was to Au1, the more
 414 labeled neurons it contained. For the ipsilateral cortex, the relationship of the FLN to the
 415 distance between the center of a projecting area and the injection site is plotted in Figure
 416 1D. There was a correlation between the projection weights of the ipsilateral cortical areas
 417 and the distance of their centers to the center of Au1, but the rule is not absolute. Motor
 418 cortex (M2), for example, sends a stronger projection to Au1 than would be expected from
 419 its distance. Also the local input is well above the regression curve, while entorhinal
 420 cortices (LEnt, MEnt) and primary somatosensory cortices (S1, S1Sh, S1BF) show weaker
 421 connections than expected. Nonetheless, this distance effect is reflected in the fact that
 422 89.0 ± 5.0 % of all labeled cells of the same cortical hemisphere were found within about
 423 1 mm of the injection site. All these labeled cells lay in the four cortical areas closest to the
 424 injection site (Au1, AuV, AuD and TeA), whose centers were within 1 mm of the injection
 425 site. This shows the existence of a dense connectivity to areas around the auditory cortex

426 and many sparse long-distance projections arising from all over the cortex in both
427 hemispheres. The binary connection density of the ipsilateral cortex amounted to 72 % (34
428 out of 47 possible cortical areas were connected to Au1). The longest of these connections
429 were about 7 mm, and originated from the ventral orbital (VO) and the frontal association
430 (FrA) cortex (Figure 1D).

431 **Fraction of supragranular layer neurons in cortex (SLN)**

432 The fraction of supragranular layer neurons (SLN) relative to all labeled neurons in a given
433 area (see Methods) was calculated for all cortical areas. These values provide a means of
434 ranking areas according to whether they were predominantly feedforward, lateral, or
435 feedback relative to Au1 (see Barone et al., 2000). Here we found that Au1 received
436 predominantly feedback connections (SLN < 0.33) from areas like visual, somatosensory,
437 motor and rhinal cortex (see Figure 1E). This was also the case for primary
438 somatosensory, insular and cingulate cortex, although the variability in the SLN of these
439 areas was high due to the small numbers of labeled cells. Even in the piriform cortex, most
440 labeled neurons were in the deepest layer, in layer 3, and it was thus classified as a
441 'feedback' area. Interestingly, SLN values in the midrange – between 0.33 and 0.66 – were
442 common. Such values indicate 'lateral' projections, which arose from areas like the
443 ipsilateral auditory cortex, the ipsilateral TeA and V2L, and from TeA, Au1 and AuV of the
444 contralateral cortex (homotopic to the injection site). Other areas with an SLN around 0.5
445 (e.g. Cg1, LPtA, MPtA) were typically represented with few labeled cells and we interpret
446 these SLN values with caution. The same caution applies to areas with an SLN of 1, where
447 only a few labeled superficial layer neurons were found (AIP, S1Sh and DLO). With these
448 exceptions, there were no projections with an SLN between 1 and 0.6, indicating that no
449 cortical area was feedforward to Au1, as one might expect for a primary sensory area. On
450 the other hand, the range of feedback and lateral projections (SLN between 0 and 0.5)

451 was well-covered. Generally, the SLN did not differ substantially between the hemispheres,
 452 except that the proportion of labeled layer 5 cells tended to be higher in the contralateral
 453 hemisphere, at the expense of layer 6 cells. But this trend was obviously not reflected in
 454 the SLN. The data and analyses are given in a single comprehensive summary diagram
 455 (Figure 2B), which includes both the SLNs and the FLNs of the network that provides the
 456 many inputs of Au1/AuV.

457 **Discussion**

458 Our analyses revealed that there are direct projections to Au1 from 98 brain areas of the
 459 mouse. The auditory cortex and the auditory thalamus provided the main weight in terms
 460 of numbers of projecting neurons, but the visual cortices, the temporal association cortex
 461 and the basal ganglia contributed substantially to the total input. Low-weight connections
 462 were found from almost every cortical area, the hippocampal formation, from many
 463 distributed nuclei in the basal forebrain, the hypothalamus, some nuclei of the thalamus,
 464 and the midbrain. Although the FLNe values varied between animals, the main inputs to
 465 Au1 were consistent. Large standard deviations were seen in areas with lower weights,
 466 simply because such areas did not contain labeled neurons in all animals. Inter-animal
 467 differences may be due to slight differences in the size and/or location of the injection site,
 468 or to actual connection weight differences between individuals.

469 **Comparison with other mouse connectome studies**

470 The Allen Brain Institute has investigated mouse brain connectivity on a grand scale using
 471 recombinant AAV as an anterograde tracer (Oh et al., 2014). We searched this database
 472 (<http://connectivity.brain-map.org/>) for the brain areas where we found retrogradely labeled
 473 cells and checked if injection of AAV into these areas had resulted in anterograde axon
 474 labeling in Au1. Indeed almost all projections to Au1 were confirmed in wildtype C57BL/6J
 475 mice. Only a few areas that we found to project to Au1 were either not injected with AAV
 476 (e.g. FrA, MGP, SNL, fiber tracts) or could not be distinguished from nearby areas due to
 477 the injection size or precision (e.g. Cg/RS, MPtA, SubB, lateral thalamic nuclei, ZID, ZIV,
 478 TC, hippocampal layers, several amygdaloid nuclei).

479

480 A similar pattern of cortical sites projecting to Au1 is evident in the iConnectome map

(<http://www.mouseconnectome.org/>), which is another growing database of mouse connectivity based on co-injections of anterograde and retrograde tracers (Zingg et al., 2014). Our findings agree with the high degree of connections between sensory modalities described in Zingg and colleagues' (Zingg et al., 2014) medial sub-network of the mouse cortex. Additionally, we found direct projections from the lateral sub-network (piriform and insular cortex) to Au1, implying that these sub-networks do not operate independently.

The existence of direct projections from the caudate putamen and globus pallidus to the neocortex has been disputed (see e.g. Divac et al., 1987), but consistent with our observations, the Allen brain atlas (<http://connectivity.brain-map.org/>) recorded that sparse anterograde labeling was indeed present in all layers of the ipsilateral Au1 and mostly in deep layers 5 and 6 in a mouse brain with AAV injection in the dorsal part of the caudate putamen. Interestingly, retrograde tracer studies in the cat point to the auditory cortex as one of the main targets of direct connections from striatum and pallidum to neocortex (Jayaraman, 1980; Olesko and Maisky, 1993). Although some cells in the pallidum that project to the neocortex might be interpreted as belonging to a cell population of neighboring brain areas (Divac et al., 1987), this does not hold in all cases. In the rat for instance, a separate population of small globus pallidus neurons projecting to the frontal cortex was described that was distinct from the larger cholinergic cells along the boundaries of the globus pallidus (Van Der Kooy and Kolb, 1985). In the mouse, a similar picture emerges: After small tracer injections, only one-directional connections from the neocortex to the striatum were reported for the ultrasonic field of Au1 (Hofstetter and Ehret, 1992).

The iConnectome study was performed using conventional retrograde tracers (Cholera

toxin B, Fluorogold), so the similarity in pattern of label suggests that the rabies virus is taken up by the same population of axons. The major advantage of the rabies viral tracers over other retrograde tracers is its sensitivity, but the negative aspect of virus tracing compared to conventional tracers is its cytotoxicity. We used the less cytotoxic AAV virus to give us an estimate of the likely maximum extent of the modified rabies uptake zone. Since the AAV reached the AuV ventral of the main injection site into Au1, we cannot completely exclude the possibility that some of the labeled cells also project to AuV. The dense labeling in the MGv, which mainly targets the primary auditory cortex (Winer et al., 1999), indicates that the rabies virus was largely confined to Au1.

Comparing species

We found a lower proportion of labeled neurons (26.5 %) within Au1 than was reported for cat Au1 (60 %, Lee and Winer, 2011) or macaque visual cortex (80 %, Markov et al., 2011). This difference is likely due to the fact that the size of the injection site was large relative to the size of Au1 in the mouse. A different trend was observed for the main thalamic input, where we found 35.5 % of extrinsic projections coming from the MGv alone. The same projection in the cat was determined to be 18 % (Lee and Winer, 2011). Thalamic input to the macaque cortex appears to be even lower by comparison (1 % to V1, Markov et al., 2011). In the Mongolian gerbil, auditory subcortical afferents formed 27 % of the total (Budinger and Scheich, 2009), compare the 43 % we found in the mouse.

525

Overall, the range of FLNe values spanned four orders of magnitude in the mouse, while in the macaque it spanned five orders of magnitude (Markov et al., 2012). The FLNe of the ipsilateral cortex was exponentially dependent on physical distance: 90 % of these cells were within 1 mm to the injection site (compared to 75 % of labeled cells within ca. 1 mm and 95 % of labeled cells within ca. 2 mm of V1 in the macaque, (Markov et al., 2011)).

Small-world networks have been a popular graph-theoretical model of cortical networks (Watts and Strogatz, 1998; Bullmore and Sporns, 2009; Budd and Kisvarday, 2012). The typical features of such networks, viz. high local clustering and short path lengths due to sparse long-range connections, optimize the integration and segregation of information and have been seen as suitable description for cortical networks. However, the high binary connectivity found in the long distance connections in macaque (66 %, Markov et al., 2012) and in the mouse (72 %) cortex is inconsistent with that of a classical small-world architecture, as emphasized by Markov and Kennedy (2013).

Cortical hierarchy

The SLN provides an index of hierarchical distance (Markov and Kennedy, 2013). ‘Lateral’ connections, which indicate that the areas lie at the same level of the hierarchy ($SLN \approx 0.5$), may thus have been expected from auditory cortices and even from ipsilateral V2L and V1, but surprisingly, the association area TeA (of both hemispheres) also connected to Au1 in a lateral fashion. As in the macaque, the immediately neighboring areas of Au1 had SLN values around 0.5. The SLN gradually declined for the more distant areas (Ect, V1, S2), but did not correlate with physical distance. Anterograde tracing with AAV virus from these areas providing lateral connections shows very sparse projections to Au1 (<http://connectivity.brain-map.org>). The axons innervate all layers, but are particularly sparse in layer 4. This projection pattern is evident even if only neurons in superficial layers or in layer 5 expressed the fluorescent label in a Cre-dependent manner (see <http://connectivity.brain-map.org>).

552

Long-distance feedforward paths from cortical areas to Au1 were notably absent, while feedback connections (from rhinal, insular, visual, somatosensory, motor and cingulate cortex) dominated. By contrast, after injections of a Cre+ pseudotyped lentivirus into the

auditory cortex of transgenic (Cre-dependent tdTomato-expressing) mice, Nelson and colleagues found that retrogradely labeled cell bodies were located preferentially in superficial layers of M1 and distributed over superficial and deep layers of M2 (Nelson et al., 2013). In that same study, however, anterogradely labeled axons in Au1 terminated mainly in layers 1 and 6, which is a typical feedback pattern according to the criteria formulated by Felleman and Van Essen (Felleman and Van Essen, 1991). An attempt to reconcile these findings with our results may be the different sensitivity of the viral tracers used or the fact that Nelson and colleagues injected more dorsally in the auditory cortex. While cortical feedforward connections were mostly absent, the primary thalamic feedforward projection from the MGV had a very large FLNe (approximately 35 %). Together with the finding that thalamic axons target proximal dendrite segments (Richardson et al., 2009), our results suggest that in the rodent Au1 cortical processing is primarily feedforward-driven. This is in sharp contrast to the picture that has emerged from the cat and primate visual cortex, where thalamic input is a very small fraction (approximately 1 %) of all the excitatory input.

Functional implications

Our results indicate that the auditory cortex is not the monosensory area its name implies, but that multimodal integration could occur even at this early stage of cortical processing. Multisensory integration is widespread in the neocortex (Schroeder et al., 2003; Clavagnier et al., 2004; Ghazanfar and Schroeder, 2006; Budinger and Scheich, 2009) and the connection between auditory and visual cortex has received particular attention. In humans, striking illusions like the McGurk effect demonstrate the interaction of auditory and visual information to form a consistent percept (McGurk and MacDonald, 1976). In the mouse, the projection from V2L was shown to modulate Au1 activity (Banks et al., 2011), but what role this visual feedback plays in auditory perception is still unclear. Activity in the

581 monkey auditory cortex is suppressed before and during certain movements, like those
 582 involved in vocalization (e.g. Müller-Preuss and Ploog, 1981; Eliades and Wang, 2003).
 583 This motor-auditory crosstalk at the cortical level may be a crucial component of auditory
 584 processing even in the mouse, which has a rich vocal repertoire. The FLN values for many
 585 of these projections are very small, which raises the pertinent question of how these few
 586 fibers exert any significant effect in Au1. The possibilities are that they act in synchrony or
 587 form very specific connections with a subset of 'pointer' neurons that control the gain of the
 588 recurrent connections in the local microcircuit of Au1 (Douglas and Martin, 2007). The
 589 anatomical weight, as measured here in the FLN, has to be translated into a functional and
 590 dynamic projection weight, which of course may differ even between areas with the same
 591 FLNe value.

592 **Conclusion**

593 Studies of anatomical 'hardwiring' provide valuable information about the network
 594 properties of interareal brain connections and provide a basis for interpreting the results of
 595 physiological and behavioral studies. The relative simplicity of measuring SLN and FLN
 596 provides a very powerful method of comparison between areas, systems, and species.
 597 Our quantification of input weights has revealed similarities to the macaque data in that the
 598 major cortical connections with auditory cortex come from adjacent areas. The pattern in
 599 the mouse also differs substantially from the macaque in having a much higher weight of
 600 connections from the thalamus and in receiving direct input from a larger proportion of
 601 cortical regions than is evident in the macaque. This difference may indicate constraints of
 602 processing in small brains, or adaptive specializations that are more species-specific.

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607

608 **Conflict of Interest**

609 The authors declare no competing financial interests.

610

611 **Role of Authors**

612 All authors were involved in all aspects of the study.

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Abbreviation	Brain area
AAD	anterior amygdaloid area, dorsal part
AAV	anterior amygdaloid area, ventral part
AIP	agranular insular cortex, posterior part
AM	anteromedial thalamic nucleus
AMV	anteromedial thalamic nucleus, ventral part
APT	anterior pretectal nucleus
APTD	anterior pretectal nucleus, dorsal part
APTV	anterior pretectal nucleus, ventral part
AStr	amygdalostratial transition area
Au1	primary auditory cortex
AuD	secondary auditory cortex, dorsal area
AuV	secondary auditory cortex, ventral area
AV	anteroventral thalamic nucleus
B	basal nucleus (Meynert)
BIC	nucleus of the brachium of the inferior colliculus
BMP	basomedial amygdaloid nucleus, posterior part
Cg/RS	cingulate/retrosplenial cortex
Cg1	cingulate cortex, area 1
Cg2	cingulate cortex, area 2
Cl	claustrum
cp	cerebral peduncle, basal part
CPu	caudate putamen (striatum)
DEn	dorsal endopiriform cortex
DI	dysgranular insular cortex
DLO	dorsolateral orbital cortex
DM	dorsomedial hypothalamic nucleus
DpMe	deep mesencephalic nucleus
DRC	dorsal raphe nucleus, caudal part
ec	external capsule
Ect	ectorhinal cortex
Eth	ethmoid thalamic nucleus
FrA	frontal association cortex
GI	granular insular cortex
HDB	nucleus of the horizontal limb of the diagonal band
ic	internal capsule
IGL	intergeniculate leaf
LDVL	laterodorsal thalamic nucleus, ventrolateral part
LEnt	lateral entorhinal cortex
LGP	lateral globus pallidus
LH	lateral hypothalamic area
LO	lateral orbital cortex
LPLR	lateral posterior thalamic nucleus, laterorostral part
LPMR	lateral posterior thalamic nucleus, mediorostral part
LPtA	lateral parietal association cortex
M1	primary motor cortex
M2	secondary motor cortex
MEnt	medial entorhinal cortex
MGD	medial geniculate nucleus, dorsal part
MGM	medial geniculate nucleus, medial part
MGP	medial globus pallidus
MGV	medial geniculate nucleus, ventral part
MO	medial orbital cortex
MPtA	medial parietal association cortex
MZMG	marginal zone of the medial geniculate
Or	oriens layer of the hippocampus
PaS	parasubiculum
PF	parafascicular thalamic nucleus
PIL	posterior intralaminar thalamic nucleus
Pir	piriform cortex

PLi	posterior limitans thalamic nucleus
Po	posterior thalamic nuclear group
PoT	posterior thalamic nuclear group, triangular part
PRh	perirhinal cortex
PrL	prelimbic cortex
PrS	presubiculum
Py	pyramidal cell layer of the hippocampus
Re	reuniens thalamic nucleus
RPF	retroparafascicular nucleus
RSA	retrosplenial agranular cortex
RSG	retrosplenial granular cortex
Rt	reticular thalamic nucleus
S	subiculum
S1	primary somatosensory cortex
S1BF	primary somatosensory cortex, barrel field
S1DZ	primary somatosensory cortex, dysgranular region
S1FL	primary somatosensory cortex, forelimb region
S1HL	primary somatosensory cortex, hindlimb region
S1Sh	primary somatosensory cortex, shoulder region
S1Tr	primary somatosensory cortex, trunk region
S2	secondary somatosensory cortex
SG	suprageniculate thalamic nucleus
SI	substantia innominata
SNL	substantia nigra, lateral part
SNR	substantia nigra, reticular part
st	stria terminalis
STh	subthalamic nucleus
Sub	submedial thalamic nucleus
SubB	subbrachial nucleus
SubI	subincertal nucleus
SuMM	supramammillary nucleus, medial part
TC	tuber cinereum area
Te	terete hypothalamic nucleus
TeA	temporal association area
V1	primary visual cortex
V2L	secondary visual cortex, lateral area
V2ML	secondary visual cortex, mediolateral area
V2MM	secondary visual cortex, mediomedial area
VEn	ventral endopiriform nucleus
VM	ventromedial thalamic nucleus
VO	ventral orbital cortex
VPM	ventral posteromedial thalamic nucleus
VRe	ventral reuniens thalamic nucleus
ZI	zona incerta
ZID	zona incerta, dorsal part
ZIV	zona incerta, ventral part

Table 1: Alphabetic list of brain area abbreviations based on the mouse brain atlas (Paxinos and Franklin 2001).

614 **Figure Legends**

615 **Figure 1**

616 **Example of an injection site.**

617 **A.** Coronal section through the injection site. The brightly labeled neurons have
618 retrogradely transported the mCherry⁺ rabies virus to their somata and dendrites. Note the
619 labeling in the cortex surrounding the injection site and in the thalamus.

620 **B.** The same coronal section showing cells labeled with NeuN. At the injection site, the
621 tissue is slightly damaged. The asterisk (*) denotes the center of the injection site. Scale
622 bars 1 mm.

623 **C. Mean FLNe ('fraction of labeled neurons extrinsic') values.** The FLNe, or input
624 weight, is shown on a logarithmic scale for all brain areas with labeled cells. Cortical areas
625 are written in bold. The projections from the contralateral hemisphere (grey triangles) are
626 generally lower and sparser than from the ipsilateral hemisphere (black circles), but follow
627 a similar pattern of relative weights. Error bars denote the relative standard deviation.

628 **D. Distance-dependence of FLN of the ipsilateral cortex.**

629 Mean FLN values sorted according to the distance of area centers to the injection site. Ca.
630 90 % of labeled neurons in the ipsilateral cortex were found within 1 mm distance, while
631 the longest projections spanned 7 mm. Average FLN values and standard deviations are
632 plotted as a function of distance to the primary auditory cortex. The curve is an exponential
633 fit: $FLN = 17.76 * \exp(-1.457 * \text{distance})$ (R-square: 0.93). Areas above the curve show
634 relatively stronger projection weights for their distance. Au1: primary auditory cortex. FLN:
635 fraction of labeled neurons.

636 **E. Mean SLN (fraction of labeled supragranular layer neurons) values of cortical**
637 **areas.** Cortical areas were sorted based on the mean SLN values, from purely
638 feedforward (SLN=1) to feedback (SLN=0), with standard deviations. Note that apart from

three exceptions, all areas project in a lateral or feedback fashion to Au1, i.e. with SLN values between 0.5 and 0. Since the piriform cortex (Pir, marked by an asterisk) is part of the three-layered archicortex, its SLN could not be determined and was set to 0 because most of the labeled cells were found in the deep layer 3. Other areas without a dark bar had an SLN of 0. Areas without error bars were either represented by one animal only or had identical SLNs in the different animals. In the contralateral hemisphere, fewer areas are shown because fewer areas contained labeled cells. For area abbreviations see Table 1.

647

648 **Figure 2**

A. Distribution of labeled cells in one brain. Coronal sections containing labeled neurons (in magenta) and stained for NeuN (in green). Sections are ordered from posterior to anterior from the top left to the bottom right. Numbers below the sections correspond to the distance from Bregma (positive values are anterior from Bregma). If only one hemisphere is shown, it is the hemisphere ipsilateral to the injection. White arrows point out single cells or group of cells in temporal association area, rhinal, visual, auditory, somatosensory, motor and orbital cortices as well as in the inferior colliculus, the auditory thalamus and the striatum. Borders of subcortical areas are only delineated if they contain retrogradely labeled neurons or are adjacent to such an area. Am: amygdala; For other area abbreviations see Table 1. Scale bars 1 mm.

B. Overview of FLNe and SLN values. The areas are arranged according to hemispheres and their approximate antero-posterior and medio-lateral position. Colors indicate SLN (blue corresponding to feedforward and dark red to feedback connections), line thicknesses correspond to the FLNe. The dots represent different mice; filled dots indicate a labeled area in the corresponding mouse.

664 FLNe: extrinsic fraction of labeled neurons. SLN: fraction of labeled supragranular layer
665 neurons. For area abbreviations see Table 1.

666

Figure 1

Figure 2