

Specific and comprehensive genetic targeting reveals brain-wide distribution and synaptic input patterns of GABAergic axo-axonic interneurons

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Abstract

Axo-axonic cells (AACs), also called chandelier cells (ChCs) in the cerebral cortex, are the most distinctive type of GABAergic interneurons described in the neocortex, hippocampus, and basolateral amygdala (BLA). AACs selectively innervate glutamatergic projection neurons (PNs) at their axon initial segment (AIS), thus may exert decisive control over PN spiking and regulate PN functional ensembles. However, the brain-wide distribution, synaptic connectivity, and circuit function of AACs remains poorly understood, largely due to the lack of specific and reliable experimental tools. Here, we have established an intersectional genetic strategy that achieves specific and comprehensive targeting of AACs throughout the mouse brain based on their lineage (*Nkx2.1*) and molecular (*Unc5b*, *Pthlh*) markers. We discovered that AACs are deployed across essentially all the pallium-derived brain structures, including not only the dorsal pallium-derived neocortex and medial pallium-derived hippocampal formation, but also the lateral pallium-derived claustrum-insular complex, and the ventral pallium-derived extended amygdaloid complex and olfactory centers. AACs are also abundant in anterior olfactory nucleus, taenia tecta and lateral septum. AACs show characteristic variations in density across neocortical areas and layers and across subregions of the hippocampal formation. Neocortical AACs comprise multiple laminar subtypes with distinct dendritic and axonal arborization patterns. Retrograde monosynaptic tracing from AACs across neocortical, hippocampal and BLA regions reveal shared as well as distinct patterns of synaptic input. Specific and comprehensive targeting of AACs facilitates the study of their developmental genetic program and circuit function across brain structures, providing a ground truth platform for understanding the conservation and variation of a bona fide cell type across brain regions and species.

eLife assessment

The authors develop a novel genetic strategy for specific and comprehensive labeling of axo-axonic cells, also referred to as chandelier cells, in the mouse brain. The approach and analysis are rigorous such that the data **convincingly** support the key conclusions, including the expanded distribution of axo-axonic cells throughout the brain. This study provides **important** new information about the distribution of a significant neuronal cell type, as well as new tools for future studies. This work will be of broad interest to neuroscientists who work on the anatomical and functional organization of neural circuits.

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Introduction

The precision of inhibitory control is crucial for regulating information processing and routing in brain circuits (Roux & Buzsáki, 2015 [↗](#)). In the cerebral cortex, inhibitory signaling is mediated by a large array of GABAergic interneurons (INs), which regulate the input and output of glutamatergic projection neurons (PNs), together forming dynamic ensembles of information processing and relay. Cortical INs comprise highly diverse subpopulations distinguished by their anatomical and physiological properties and gene expression profiles (Fishell & Kepecs, 2020 [↗](#); Gouwens et al., 2020 [↗](#); Huang & Paul, 2019 [↗](#); Tremblay et al., 2016 [↗](#)). Recent single-cell RNAseq and computational analyses have delineated the hierarchical tree-like organization of cortical GABAergic neurons from subclasses and supertypes down to transcriptomic subtypes (Tasic et al., 2018 [↗](#))(BICCN 2021). Among these, the axo-axonic cells (AACs), or chandelier cells (ChCs), represent the most distinctive and a bona fide type, as they exclusively innervate the axon initial segments (AIS) of pyramidal neurons (PNs) (Somogyi, 1977 [↗](#); Somogyi et al., 1982 [↗](#)). Because AIS are the site of action potential initiation and individual AACs innervate hundreds of PNs in their vicinity, AACs are thought to exert decisive control over spike generation in ensembles of PNs (Crick 1986) (Howard et al., 2005 [↗](#)), thereby regulating network operations and information relay. Thus, a more complete understanding of AACs will not only provide key insights into the functional organization of cortical circuits but also facilitate an integrated multi-modal definition of ground truth neuron types, shedding light on the granularity of hierarchical cell type organization. However, current knowledge on AAC distribution, connectivity, and function remains incomplete, largely due to the lack of reliable experimental tools.

An ideal genetic tool for studying AACs, or any other bona fide neuron type, would have two important features: specificity (targeting only AACs but no other cells) and comprehensiveness or even completeness (capturing most if not all AACs). Genetic labeling of AACs was first achieved through fate mapping neural progenitors of the medial ganglionic eminence (MGE) at late embryonic stages using the *Nkx2.1-CreER* mouse line (Taniguchi et al., 2013 [↗](#)). This approach has facilitated analyses of neocortical AAC subtypes (Wang et al., 2019 [↗](#)), transcriptome profiles (Paul et al., 2017 [↗](#)), connectivity (Lu et al., 2017 [↗](#)), synapse development (Ishino et al., 2017 [↗](#); Tai et al., 2019 [↗](#)), and activity-dependent pruning (Wang et al., 2021 [↗](#)); it also enabled a comprehensive fate mapping of AACs across MGE neurogenesis (Kelly et al., 2019 [↗](#)). However, late embryonic induction in *Nkx2.1-CreER* mice is not only cumbersome but also not entirely specific to AACs (Kelly et al., 2019 [↗](#)), and does not capture all AACs. With the identification of several putative AAC molecular markers from scRNAseq datasets (Paul et al., 2017 [↗](#); Tasic et al., 2018 [↗](#)), several gene-knockin driver lines have been generated, which have facilitated studying AACs. Yet, none of these driver lines have been shown to be specific and comprehensive. For

example, the *Vipr2-ires-Cre* driver captures a subset of neocortical and basolateral amygdala (BLA) AACs, but is not entirely specific in these areas (Daigle et al., 2018 [↗](#); Nakashima et al., 2022 [↗](#); Tasic et al., 2018 [↗](#)). Similarly, our *Unc5b-CreER* line enabled AAV targeting of hippocampal AACs, but also labels endothelial cells when combined with a reporter line (Dudok et al., 2021 [↗](#)). Despite extensive effort to date, no singularly unique molecular marker for AACs has been identified. Indeed, the same is true for most if not all neuronal subtypes of the mammalian brain. Therefore, specific and comprehensive genetic access to ground truth neuron types remains a broad and major challenge in neural circuit studies.

Here, we leverage lineage origin and molecular markers to establish an intersectional strategy, achieving highly specific and comprehensive targeting of AACs across the mouse brain. We discovered that much beyond the neocortex, hippocampus and BLA as previously described, AACs are in fact widely distributed across all pallium-derived brain structures and beyond. These include not only the dorsal pallium-derived neocortex and medial pallium-derived hippocampal formation, but also the lateral pallium-derived claustrum-insular complex, the ventral pallium-derived extended amygdaloid complex, and olfactory centers such as piriform cortex. In addition, putative AACs are also abundant in anterior olfactory nucleus, taenia tecta and are also found in the lateral septum and the diencephalic or third-ventricle-derived hypothalamus. The comprehensive labeling of cortical AACs allowed us to quantify their areal, laminar, and axon terminal distribution as well as to describe multiple anatomic subtypes. We further extend our strategy to AAV-based targeting and map synaptic input sources to AACs in sensorimotor cortical areas, hippocampal CA1, and BLA, demonstrating feasibility for functional manipulations. Together, these results provide a brain-wide overview of AACs and their subtype variations and input sources in the cerebral cortex. Precise and comprehensive genetic access to AACs sets the stage for understanding many aspects of their role in circuit development and function across the extended brain regions. This establishes a powerful model system to study the conservation and divergence of a ground truth neuron type across different brain regions towards understanding the principles of cell type organization.

Results

Lineage and marker-based intersectional targeting captures AACs across brain regions

The embryonic subpallium generates GABAergic neurons of the telencephalon, with the medial ganglionic eminence (MGE) giving rise to AACs (Kelly et al., 2019 [↗](#); Taniguchi et al., 2013 [↗](#)) while other progenitor domains, such as the caudal ganglionic eminence (Miyoshi et al., 2015 [↗](#)) or preoptic area (Gelman et al., 2011 [↗](#)) do not. Within the MGE *Nkx2.1* lineage, AACs are generated in two consecutive waves during both early and late neurogenesis (Kelly et al., 2019 [↗](#)), extending our initial report of their birth pattern (Taniguchi et al., 2013 [↗](#)). Therefore, late embryonic induction in the *Nkx2.1-CreER* line only enriches a subset of neocortical AACs and also labels at least two other interneuron types (Kelly et al., 2019 [↗](#)); this strategy also misses AACs in the hippocampus, BLA, and potentially other brain areas that are born earlier (Kelly et al., 2019 [↗](#)). Taking a different approach based on scRNAseq analysis, we identified several putative AAC markers (Paul et al., 2017 [↗](#)) and accordingly generated *Unc5b-CreER* and *Pthlh-Flp* driver lines. However, neither by itself is fully specific to AACs: the former also labels endothelial cells and sparse cortical pyramidal neurons (data not shown), and the latter labels a subset of CGE-derived interneurons (Figure 1—figure supplement 3m-p [↗](#)). To purify AACs from these driver lines, we then designed an intersectional strategy that combines lineage and molecular markers (Figure 1a [↗](#)); we tested the intersection of *Nkx2.1-Flp* with *Unc5b-CreER* as well as *Nkx2.1-Cre* with *Pthlh-Flp*. We expected that the constitutive *Nkx2.1-Flp* or *Cre* line would cover the entire output from MGE, including all AACs, and the intersection with postmitotic markers would specifically capture AACs.

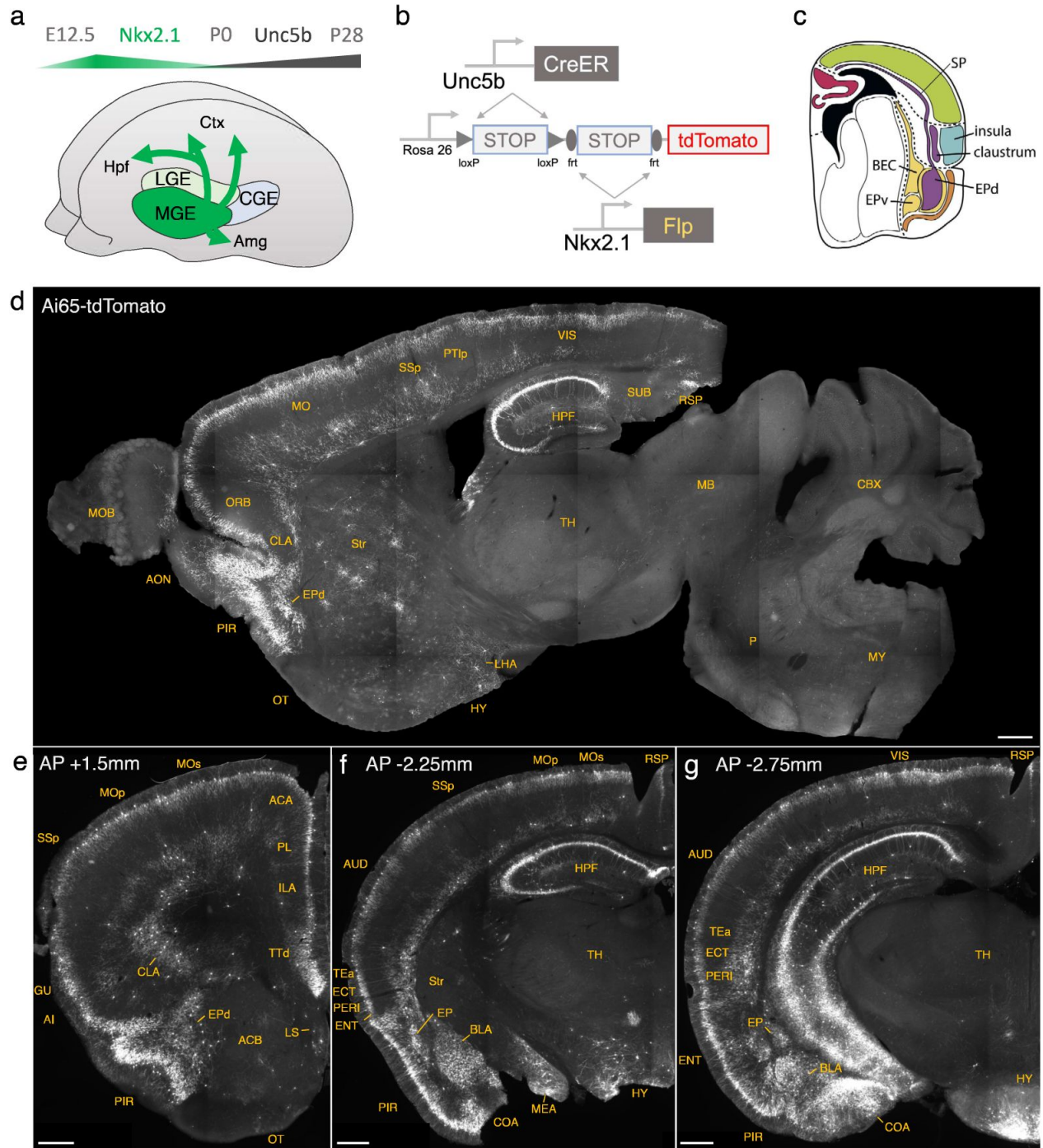


Figure 1.

Developmental genetic intersectional targeting reveals brain-wide AAC distribution patterns.

a) Schematic of lineage (*Nkx2.1*) and marker (*Unc5b*) intersection for pan-AAC labeling. Embryonic *Nkx2.1* expression defines MGE interneuron identity while subsequent postnatal *Unc5b* expression restricts interneurons to AACs. Height of green or gray shade represents relative expression levels.

b) Configuration of triple allele intersectional labeling, combining *Unc5b-CreER* and *Nkx2.1-Flp* drivers and the Cre-AND-Flp *Ai65* reporter. Embryonic Flp expression removes the frt-flanked STOP cassette and postnatal CreER induction removes the loxp-flanked STOP, thereby activating constitutive tdTomato expression.

c) Schematic showing that MGE-derived AACs migrate and populate all four pallial-derived brain structures depicted in color: medial pallium (red), dorsal pallium (green), lateral pallium (violet and blue), and ventral pallium (yellow and orange). Adapted from [Puelles, 2017](#).

d) Representative midsagittal section showing AAC labeling in cerebral cortex, hippocampal formation (HPF), and olfactory centers such as piriform cortex (PIR) and anterior olfactory nucleus (AON). Note sparse labeling in lateral hypothalamus (LH) and striatum (str).

e-g) Representative coronal sections at specified anterior-posterior coordinates (from Bregma) showing dense AAC labelling in cerebral cortex (e-g), HPF (f-g), claustrum (CLA, e), endopiriform (EP, f-g), taenia tecta (TTd, e), lateral septum (LS, e), basolateral amygdala (BLA, f-g), cortical amygdala (COA, f-g), medial amygdala (MeA, f), and hypothalamus (HYP, g). (Scale bars, 500µm.) Abbreviations for anatomical structures are listed in Appendix. All images showing Ai65-tdTomato were immunostained for signal amplification.

We first combined *Nkx2.1-Flp* and *Unc5b-CreER* driver lines with an *Ai65* intersectional reporter allele to generate *Unc5b-CreER;Nkx2.1-Flp;Ai65* mice (**Figure 1b**). Tamoxifen induction (TM) allows dose-dependent titration of AAC labeling density, allowing the characterization of global distribution patterns *en masse* with high dose as well as the evaluation of individual cell morphologies with sparser labeling by lower dose (see methods). Indeed, postnatal high dose TM induction generated dense and highly characteristic AAC labeling patterns throughout the neocortex, hippocampus, and BLA (**Figure 1d-g**). The characteristic chandelier axon arbor morphology with strings of synaptic boutons (“cartridges”) along the AIS of pyramidal neurons unequivocally indicated their AAC identity (**Figure 1**, **Figure 1—figure supplement 1-3**).

Surprisingly, cell labeling extended much beyond these three previously reported structures that contain AACs. As the AAC identity of labeled cell are validated in most but not all these structures, we tentatively call some of these putative AACs (pAACs). Extensive analysis of these brain regions revealed an overarching pattern: AACs and pAACs are abundantly present in essentially all pallium-derived structures and several additional structures (**Table 1**; **Figures 1c-g**, **Figure 1—figure supplement 1-2**, Supplemental movie 1). The dorsal pallium (dPAL)-derived structures include the entire neocortex and entorhinal/ectorhinal cortices. The medial pallium (mPAL)-derived structures include the entire hippocampal formation. Beyond these previously known structures, pAACs are abundant in lateral pallium (lPAL)-derived claustrum, insular, and endopiriform dorsal. In addition, the ventral pallium (vPAL)-derived structures include most of the amygdaloid complex (cortical amygdala, basolateral and basomedial amygdala, and medial amygdala) and extended amygdala (bed nucleus of stria terminalis). Most surprisingly, AACs are highly abundant in a set of olfactory centers including piriform cortex, anterior olfactory nucleus, and taenia tecta. Among these, the piriform derives from the vPAL while AON and TTd derive from

septum neuroepithelium. Finally, pAACs are found in lateral septum (also derived from septum neuroepithelium (Magno et al., 2022 [↗](#)) and parts of the hypothalamus (derived largely from neuroepithelium of the third ventricle).

Consistent with these findings, the *Pthlh-Flp;Nkx2.1-Cre;Ai65* mice were found to have a similar overall pattern of pAAC distribution across different brain regions, with several differences (Figure 1—figure supplement 3 [↗](#)). First, this intersection also labeled a set of layer 4 interneurons in the somatosensory cortex (Figure 1—figure supplement 3f [↗](#)). Second, *Pthlh;Nkx2.1* yielded slightly higher cell targeting in retrosplenial cortex (Figure 1—figure supplement 3h-i [↗](#)). Third, *Pthlh;Nkx2.1* labelled fewer cells in the AON and BST). As an additional application of the *Pthlh-Flp* line, we demonstrate that when combined with an inducible *Nkx2.1-CreER* driver (Taniguchi et al., 2013 [↗](#)) the *Pthlh-Flp;Nkx2.1-CreER;Ai65* mice led to exclusive fate mapping of AACs (Figure 1—figure supplement 3j-l [↗](#)). The overall similarity between *Unc5b/Nkx2.1* and *Pthlh/Nkx2.1* patterns, and high density of cells captured, provided independent evidence that these intersectional approaches may have captured most if not all AACs in the brain.

In addition to targeting AACs, the *Pthlh-Flp* is a useful tool for at least two additional cell types. First, consistent with its expression in a set of CGE-derived interneurons (including VIP-expressing cells), intersection with the *VIP-Cre* driver line, which alone expresses in other cell types, was highly-specific for an interneuron subtype with striking vertical morphology that may represent interneuron-selective cells (Figure 1—figure supplement 3m-p [↗](#)). Second, *Pthlh/Nkx2.1* intersection yielded dense labeling of striatal interneurons (i.e. striatal neurons without axons in SNr/GPi) (Figure 1—figure supplement 3f-h [↗](#)), consistent with known *Pthlh* expression in striatal PV interneurons (Munoz-Manchado et al., 2018 [↗](#)).

In sum, our lineage and marker intersections combining *Nkx2.1-Flp/Cre*, or *CreER* with *Unc5b-CreER* or *Pthlh-Flp* appear to have achieved unprecedented specificity and comprehensiveness of AAC targeting. To validate this result, in the following sections, we present more detailed and quantitative descriptions of AACs and pAACs across brain structures.

Areal, laminar, and subtype distribution of AACs across the neocortex

Previous studies have identified ChCs in multiple neocortical areas and cortical layers with the observation of several morphological variants (Kelly et al., 2019 [↗](#); Somogyi et al., 1982 [↗](#); Taniguchi et al., 2013 [↗](#); Wang et al., 2019 [↗](#)), but the global and quantitative distribution of ChC across the cerebral cortex and cortical layers is unknown. To first validate the specificity of our method to ChCs, we used single low dose TM induction to sparsely label individual cells across cortical areas (Figure 2—figure supplement 1 [↗](#)). Combined with immunolabeling of AnkyrinG-stained pyramidal neuron (PN) AIS, we showed that the vast majority of RFP⁺ cells (97.3%, n=110 cells from 6 mice) had axonal cartridge structure with synaptic boutons along PN AIS, validating their ChC identity (Figure 2—figure supplement 1 [↗](#)).

Developmental origin	Brain structure	Evolutionary term	Validation
Dorsal pallium	neocortex all cortical areas entorhinal, perirhinal	isocortex	yes
Medial pallium	hippocampal formation dentate, CA1, CA2, CA3 subiculum	archicortex	yes
Lateral pallium	claustrum insular endopiriform dorsal		not yet
Ventral pallium	amygdaloid complex - BLA - CoA - MEA - BMA extended amygdala - CeA (no AACs found) - BNST (putative cases only) olfactory cortex - piriform - endopiriform ventral [no]	paleocortex	yes
Septal neuroepithelium	-lateral and medial septum -tanial tecta -AON		yes
Hypothalamo-telencephalic prosomere h1/h2	hypothalamus - arcuate nucleus - periventricular hypothalamic nucleus		not yet

Table 1.

Brain-wide distribution pattern of putative AACs

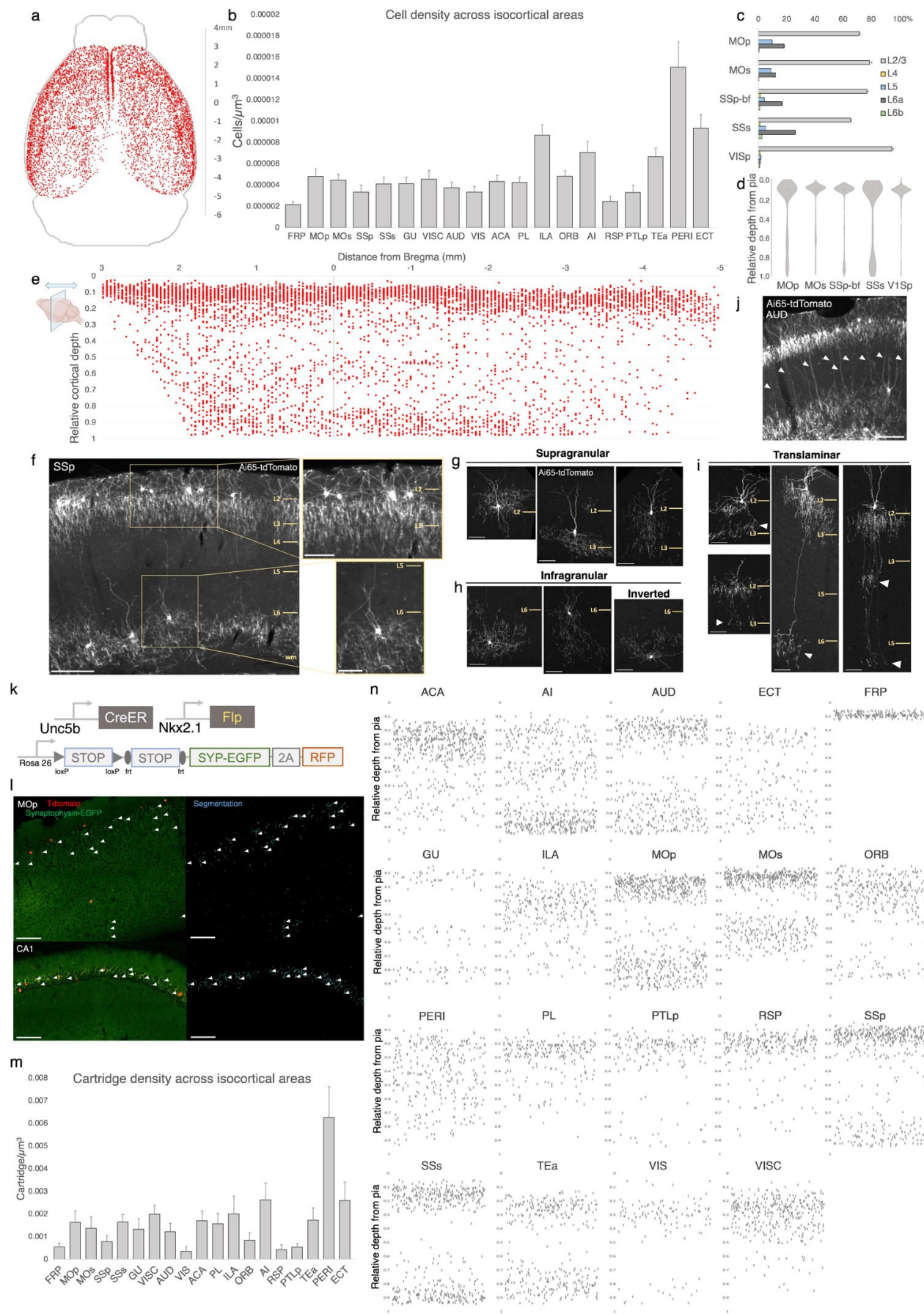


Figure 2.

Characterization of chandelier cells (ChCs, i.e. cortical AACs) in cerebral cortex.

(a) Representative 2D stereotactic plot of cortical ChC distribution in *Unc5b; Nkx2.1*. Dots represents individual RFP-labelled cells. Anterior-posterior distance from Bregma (vertical scale on right).

(b) Normalized ChC cell density (cells/ μm^3) following registration to ARAv3 isocortical areas. For b and c, data are mean $\pm$ SEM.

(c) Comparative bar plot showing relative proportion in each cortical layer in sensorimotor cortices.

(d) Violin plots of ChC cell density proportion along pia-to-white matter cortical depth in each sensorimotor cortical areas. Median is used for each violin plot.

(e) Scatterplot of ChC distribution along pia-to-white matter depth (vertical axis) and anterior-posterior distance from Bregma (horizontal axis, in mm); red dots represent individual cells. While a vast majority ($\sim 80\%$) of ChCs occupy a band near the layer I/II border, ChCs in other layers account for $\sim 20\%$ of the total cortical population and vary in depth distribution by anterior-posterior cortical location.

(f) Representative ChC laminar distribution pattern, showing largely separated supragranular (top inset) and infragranular (bottom inset) laminar subtypes. (Scale bars, $200\mu\text{m}$ and insets, $100\mu\text{m}$.)

(g-i) Multiple ChC laminar subtypes revealed by single-cell labeling using low-dose tamoxifen induction.

These include supragranular subtypes (g), infragranular and inverted subtypes (h), and translaminal L2/3 subtypes that extend long vertical axons (white arrowheads) to deep layers (i). White arrowheads highlight axonal plexi distant from soma in translaminal types. (Scale bars, $65\mu\text{m}$.)

(j) Example of regional ChC morphological diversity in auditory cortex, with relatively high proportion of translaminal subtypes. (Scale bar, $200\mu\text{m}$.)

(k-n) ChC axon terminal (i.e. “cartridge”) labeling and mapping using intersectional synaptophysin-EGFP/cytoplasmic-tdtomato reporter mouse line.

(k) Diagram of intersectional genetic labeling of ChC cartridges (EGFP) and soma (tdTomato).

(l) Pixel classifier trained to segment EGFP-expressing ChC cartridges in cortex and CA1 hippocampus. White arrowheads indicate detected EGFP-labelled cartridges. (Scale bars, $100\mu\text{m}$.)

(m) Density of ChC cartridges (cartridges/ μm^3) registered to ARAv3

(n) Representative relative depth plots (from pia) of individual detected cartridges at a single AP coordinate for each cortical area. Refer to Appendix for ARAv3 area label abbreviations. Note reduced deep layer cartridges in FRP, RSP, and V1.

To systematically map and quantify ChC distribution across the entire cerebral cortex, we then used high dose TM induction in *Unc5b-CreER;Nkx2.1-Flp;Ai65* mice to densely label ChCs and performed serial 2-photon tomography (STP) across the entire mouse brain at $50\mu\text{m}$ intervals (Supplemental movie 1). The whole brain STP datasets were then registered to the Allen CCFv3 atlas, followed by segmentation of anatomical regions and an automated cell detection algorithm was applied to identify and quantify labeled cells in each region (Ragan et al., 2012; Kim et al.,

2017; Matho et al., 2021). ChCs were detected across all areas of cerebral cortex (**Figure 2a** [↗](#); Supplemental movie 1). Normalized cell density analysis revealed area-specific patterns (**Figure 2b** [↗](#)). The highest densities were observed in ventrolateral areas such as PERI, ECT, TEA, and AI as well as ventromedially in ILA (see appendix for abbreviations). There was a slight decline in ChC density very rostrally in FRP or caudally in RSP.

We further mapped ChC distribution across cortical layers in several sensory and motor areas (**Figure 2c** [↗](#), d). The majority of ChC somas were located in L2/3, though this was area specific, with normalized density ranging from the lowest in SSs ($65.5 \pm 0.6\%$) to highest in VISp ($94.7 \pm 1.0\%$). An analysis of relative cortical depth (**Figure 2e** [↗](#)) revealed that ~80% of ChCs occupied a narrow band just below the L1/2 border, though this varied by anterior-posterior location. Nevertheless, ChCs were found at nearly all cortical depths, with a sizable proportion forming a second, less dense band above the white matter tract. This largely “bilaminar” pattern was highly evident in certain areas such as the SSp (**Figure 2f** [↗](#)).

To more systematically sample the morphological variations of ChCs, we used low dose TM induction to resolve individual cells across areas (**Figure 2—figure supplement 1** [↗](#)). We identified all the previously described laminar and morphological subtypes (Wang 2019), including supragranular (L2/3) (**Figure 2g** [↗](#)), infragranular (L5/6) (**Figure 2h** [↗](#)), and inverted (axon arbors extend above cell soma). Notably, we found rather frequent bi-laminar and tri-laminar subtypes—L2/3 ChCs extending their axon arbors not only locally but further down to another one or two deep layers (**Figure 2i** [↗](#)). These translaminar ChCs may coordinate PN ensembles between cortical layers. The abundance of translaminar ChCs varied among cortical areas, e.g. with high abundance in auditory cortex (**Figure 2j** [↗](#)).

In addition to soma position and cell morphology, we sought to map the distribution of ChC axon terminals (i.e. cartridges) throughout the mouse cortex. For this purpose, we used a Cre/Flp-dependent reporter line expressing a synaptophysin-EGFP fusion protein and cytoplasmic tdTomato (Niederkofler et al., 2016) (**Figure 2k-l** [↗](#)). Following high-dose induction and dense labeling, we performed whole brain STP imaging and calculated cartridge density across ARAv3 areas (see Methods). ChC cartridges exhibited a wide range of densities across areas and layers (**Figure 2m** [↗](#)), with overall similarity to ChC soma densities in those areas and layers (**Figure 2b** [↗](#)). Plotting the relative laminar depth for ChC cartridges in each area (**Figure 2n** [↗](#)) revealed characteristic patterns of population-level laminar innervation onto PNs.

AACs in the hippocampus

Decades of studies of hippocampal AACs using traditional anatomical and physiological methods have implicated their role in network oscillations, including sharp waves and epilepsy (Fitzgerald et al., 2013 [↗](#); Klausberger et al., 2003 [↗](#); Viney et al., 2013 [↗](#)) but the quantitative distribution of AACs across hippocampal formation has remained unknown, and reliable genetic access to hippocampal AACs is not well established. We applied *Unc5b/Nkx2.1* intersectional targeting and STP to map AAC distribution throughout hippocampal compartments. Single cell labeling by low-dose tamoxifen and immunohistochemistry confirmed that the vast majority (98.2%, $n=56$ cells, 6 mice) were AACs, characterized by vertically-oriented cross laminar apical dendrites in CA1-CA3 and axon terminals innervating PN AIS (**Figure 3a-b** [↗](#), d). Dense labeling with high dose TM followed by cell density analysis revealed that AAC density was, surprisingly, highest in CA2 and lowest in dentate gyrus (**Figure 3c** [↗](#)), with density in CA1 and CA3 approximately equal. Only a very sparse population of AAC was labelled in dentate gyrus. Overall AAC density in CA2 was 3-4 fold greater than CA1/CA3 and 13-fold greater than DG (**Figure 3c** [↗](#)). As the density of PNs across hippocampal areas are overall similar, this result suggests substantially different AAC-PN innervation ratio and that AACs may exert much denser and likely stronger control of CA2 PNs than CA1/CA3 PNs or DG granule cells.

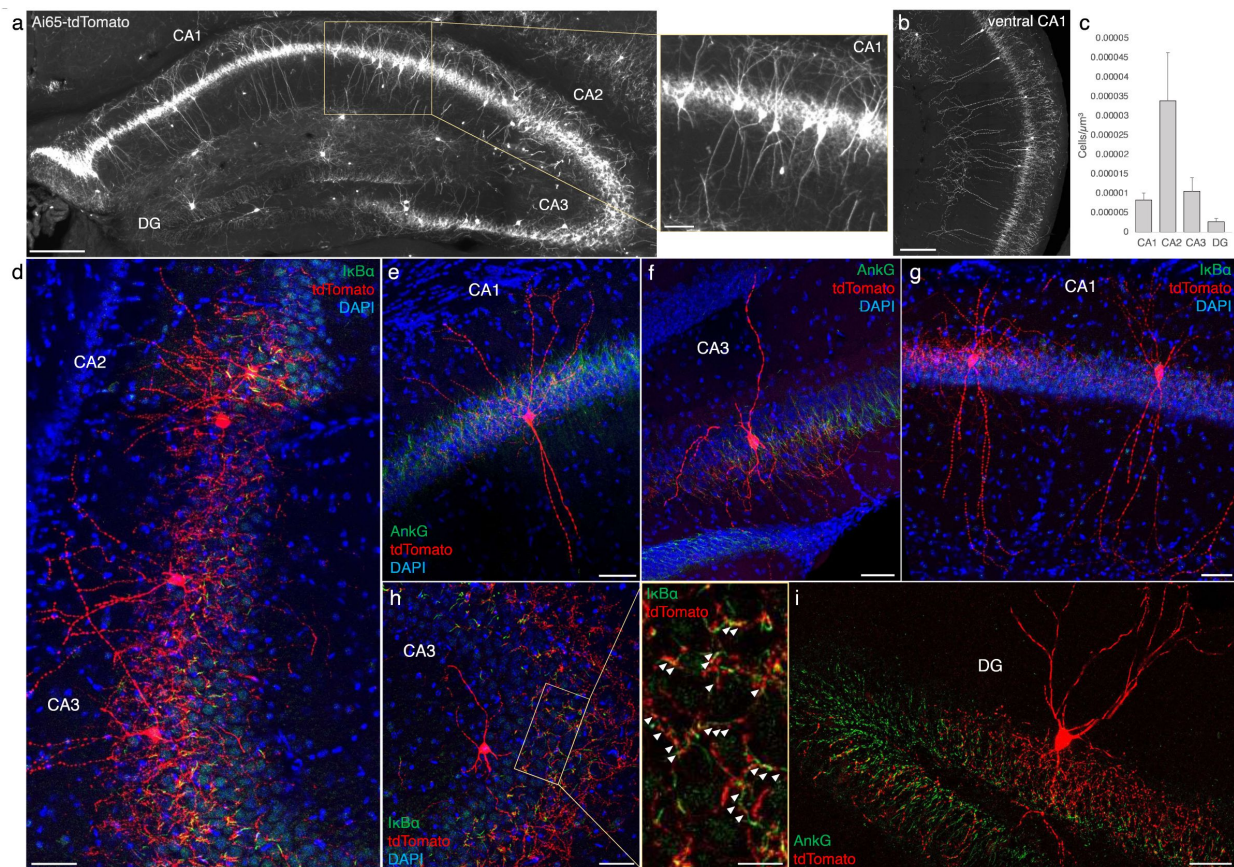


Figure 3.

Distribution and validation of AACs in hippocampus.

(a) Population labeling of AACs throughout hippocampal compartments by *Unc5b; Nkx2.1* intersection. Note the highly-stereotyped banded distribution of AACs in CA1-CA3, with somata in the stratum pyramidale and vertically oriented dendrites extending basally to the stratum oriens and apically to the stratum lacunosum-moleculare. AACs are much sparser in dentate gyrus, with individual cells having elaborate and extended horizontal axon arbor. Scale bars, 200μm and 50μm for high-magnification inset.

(b) AACs in ventral CA1 have overall similar morphology as in dorsal CA1, though with longer basal-apical dendrites matching the ventral CA1 anatomy.

(c) Normalized AAC cell density (cells/μm³) across hippocampal compartments. CA2 has 3-4-fold higher AAC cell density compared to CA1 and CA3 and 12-fold higher compared to DG.

(d-i) Immunohistochemistry validation with the AIS markers AnkG or IkBα (green) and sparse-labelled RFP cells confirm axo-axonic targeting by the intersectional strategy. Examples of hippocampal AACs in CA1 (e, g), CA2 (d) and CA3 (d, f, h). In contrast, AACs in DG have much wider axon arbor in the stratum granulosum, potentially innervating many more granule cell AIS compared to in other hippocampal compartments (i). High-magnification inset: white arrowheads indicate segments of IkBα and RFP apposition. (Scale bars, 50μm and 20μm for high-magnification inset.) For b, data are mean ± SEM.

AACs in anterior insular, claustrum, and dorsal endopiriform nuclei

According to the updated tetrapartite model of pallial/cortical development (Figure 1c), the lateral pallium gives rise to the claustrum-insular complex as well as the dorsal endopiriform nuclei (EPd) (Puelles, 2017). Our brain wide imaging of *Unc5b-CreER;Nkx2.1-Flp;Ai65* mice revealed pAACs deployed across these three regions (Figure 4a). Unlike cortical and hippocampal AACs whose axon arbors exhibit highly characteristic laminar pattern, reflecting the organization of their postsynaptic targets (i.e. AIS of PN), the axon arbors of RFP cells in AI, CLA, EPd do not show clear laminar patterns and exhibit less uniformity and are more multipolar in morphology (Figure 4b-c), making it more difficult to ascertain their identity. Nevertheless, strings of synaptic boutons were frequently identified that co-localized with AnkG labeled AIS, suggesting these as pAACs. Quantification from dense labeling revealed higher pAAC density in EPd compared to claustrum and EPv, the latter of which structure is derived from ventral pallium (Figure 4d).

AACs in the amygdaloid complex and extended amygdala

The amygdaloid complex includes over a dozen nuclei and can be segregated into 5 groups (Beyeler & Dabrowska, 2020): 1) the basolateral amygdala (BLA) divided into a dorsal section (lateral amygdala, LA) and basal section (basal amygdala, BA), 2) the basomedial amygdala (BMA), 3) the central amygdala (CeA) further splits into medial, lateral, and central sections (CeM, CeL, and CeC), 4) the medial amygdala (MeA), and 5) the cortical amygdala (CoA). Furthermore, the CeM extends rostrally and medially, thereby including the bed nucleus of stria terminalis (BST) to form the extended amygdala. Based on developmental origin, connectivity, and gene expression, these groups can be assigned into two large categories: pallium-derived cortex-like structures and subpallium-derived basal ganglia-like structures. The former includes BLA, CoA, BMA, and MeA, while the latter includes CeA and BST. Within the amygdala nuclei, projection neurons are exclusively glutamatergic in BLA, CoA, BMA, exclusively GABAergic in CeA, and predominantly GABAergic in MeA and BST. In rodents, there is also a population of GLU PNs (derived from third ventricle neuroepithelium) that populates the BST, MeA, and hypothalamus (Garcia-Moreno et al., 2010; Huilgol and Tole, 2016).

In this context, we found AACs or pAACs in all the amygdala nuclei containing GLU PNs, i.e. except CeA (Figure 4i-h). Sparse labelling and immunohistochemical co-staining with AnkG confirmed that the labeled cells in these compartments were nearly exclusively AIS-targeting (97.2%, n=36 cells, 6 mice). Morphologically, AACs in the amygdala tended to be similar to those in the claustrum, exhibiting multipolar dendrites and multipolar axon terminal branches (in contrast to axon arbor of cortical AACs that typically extend either below or above the soma) (Figure 4f). The density of AACs in amygdala appears to correlate with the known abundance of GLU PNs: densities in BLA, CoA, BMA were much higher than those in MeA and BST. Among the pallium-derived amygdala subdivisions (LA, BLA, BMA, PA), AACs were present at roughly similar densities (Figure 4i), while among striatum-like amygdala nuclei they were largely concentrated in MeA (Figure 4j). Our results indicate that AACs are deployed to all amygdala nuclei containing GLU PNs. Within the largely GABAergic structures such as MeA and BST, we hypothesize that pAACs might innervate the minor glutamatergic PNs in these structures.

AACs in olfactory centers: piriform cortex, AON, TTd

A major novel finding is the surprising abundance of AACs across multiple olfactory centers. Derived from the ventral pallium, the piriform cortex had been reported to contain axo-axonic synapses by co-localization of the inhibitory presynaptic marker GAT1 and AnkG (Wang and Sun, 2012). Our dense labelling in *Unc5b-CreER;Nkx2.1-Flp;Ai65* mice revealed a striking abundance of AACs that span the anterior-medial extent of the piriform in a single layer (Figure 5a-c). Sparse

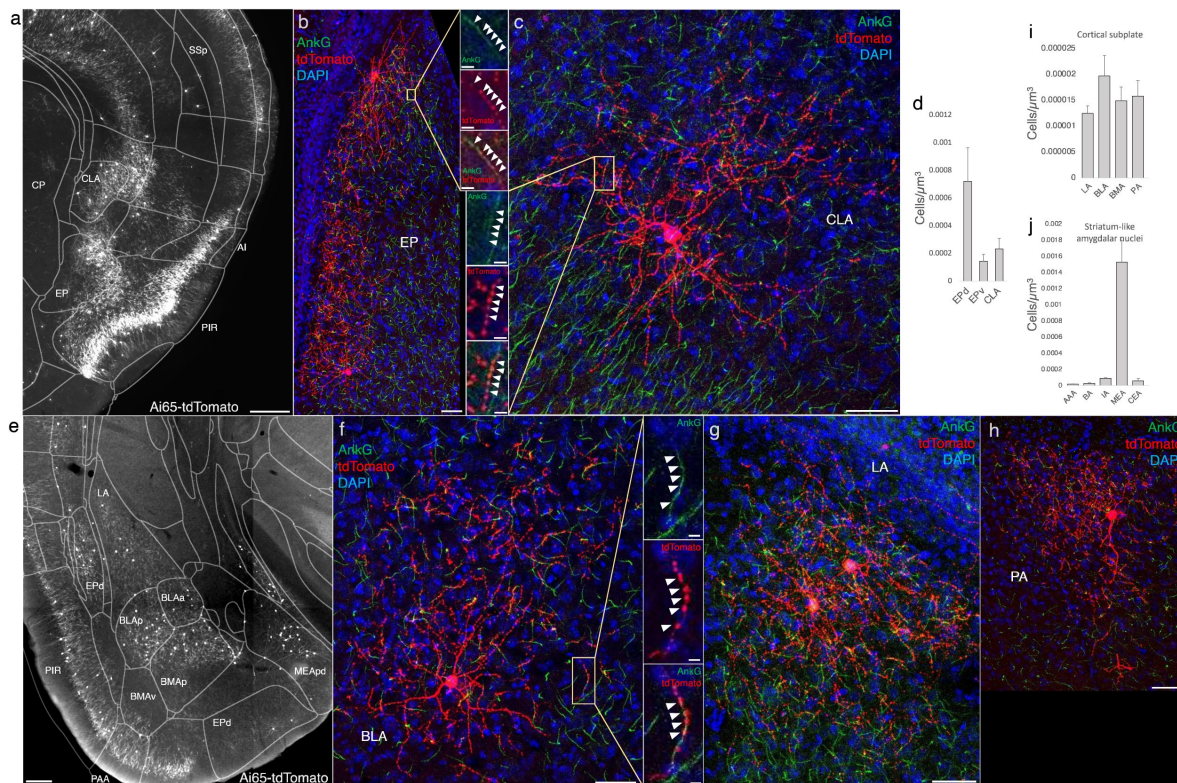


Figure 4.

Distribution of AACs across IPAL- and vPAL-derived structures

(a) Dense labelled RFP cells in claustrum, endopiriform nucleus, and insula. Note AACs in insula follow the banded laminar pattern of AACs similar to cortical upper layers and piriform, while those in claustrum and endopiriform are more dispersed in distribution.

(b-c) Sparse labeling of single cell morphologies in endopiriform nucleus (b) and claustrum (c) showing elaborate dendritic and axonal branching structure. High-magnification insets: white arrowheads indicate segments of AnkiG and RFP apposition. Scale bars, 50µm and 5µm for single cell examples and insets, respectively.

(d) Normalized AAC cell density (cells/um3) across lateral pallial structures. pAACs are approximately five-fold more prevalent in dorsal EP compared to ventral EP. Data are mean ± SEM.

(e) Dense labelling throughout vPal-derived amygdalar nuclei and piriform cortex. Note there is a degree of compartmentalization, with fewer cells in the ventral-most portions of EPd and the ventral BMA. The laminar pattern of AAC distribution in piriform and Piriform-amygdalar area (PAA) is similar to other three-layered allocortices.

(f-h) Immunohistochemistry of single cell labeling shows the multipolar morphologies characteristic of amygdalar AACs. High-magnification inset: white arrowheads indicate segments of AnkiG and RFP apposition. (Scale bars, 500 µm for grayscale panels, 50µm and 5µm for single cell examples and insets.)

(i-j) Normalized AAC cell density (cells/um3) across cortical subplate (i) and striatum-like (j) amygdala. Data are mean ± SEM. All images showing Ai65-tdTomato were immunostained for signal amplification, except for panels a and e which were native fluorescence.

labeled single AACs exhibited characteristic morphologies analogous to L2/3 AACs of the neocortex, with extended branching of apical dendrites to L1 and dense axon arbors below the cell body forming cartridges along PN AIS (**Figure 5d-j**). The single layer distribution pattern of piriform AACs continued dorsally to the insular cortex and ventrally to piriform-amygdala area (PAA) and COA (**Figure 5c**).

Furthermore, we found that AACs are deployed to two other olfactory centers that derive from outside of the four pallial domains: namely the anterior olfactory nucleus (AON) and taenia tecta (TT) (**Figure 6a-b**). Though exhibiting a range of different morphologies, AACs in all three areas resemble those of L2/3 neocortex, with apical dendrites often extending to the pia and basally-oriented axon arbors; their somata are located at the upper border of the pyramidal cell layer in the case of TT and AON (**Figure 6c-g**). Overall, AACs in piriform, TT, and AON exhibited higher density than AACs in cortex, hippocampus, and amygdala (**Figure 6h**, see Extended Data for **Figures 2**, **3**, and **6**).

AACs in the lateral septum

The LS, despite having no distinct laminar structure (delineated in ARAv3), shows some evidence of differential mRNA expression in superficial and deep domains, possibly analogous to a laminar organization (Besnard and Leroy, 2022). In *Unc5b-CreER;Nkx2.1-Flp;Ai65* mice, AACs tended to locate towards the midline (**Figure 6b**). Individual AACs were morphologically similar to cortical AACs, with extensive apical dendrites extending toward the midline and axon arbors and cartridges typically protruding away and into the cellular layer (**Figure 6i-k**).

Monosynaptic input tracing to AACs in sensorimotor cortex

In addition to using Cre AND Flp-dependent reporter lines for AAC access, it is advantageous to extend our intersectional strategy to engage viral vectors that enable more selective and anatomy-based labeling, recording, and manipulation. Because the endogenous expression of *Unc5b* and *Nkx2.1* occur at non-overlapping stages in development, it is not feasible to directly use Cre-AND-Flp dependent viral vectors (Fenno et al., 2020). Instead, we crossed *Unc5b-CreER;Nkx2.1-Flp* driver with a Cre-AND-Flp dependent tTA2 reporter line (**Figure 7a**) (Matho et al., 2021), which provides an indelible genetic handle for delivery of tTA-activated viral reporters. We tested this approach in AACs to map their synaptic input source by using tTA-activated AAVs in of *Unc5b-Cre;Nkx2.1-Flp;dual-tTA2* mice (**Figure 7b**; **Figure 7 – Figure supplement 1b, c**). We used the PV cell (PVC) population for comparison largely due to convenience, recognizing the fact that PVCs also include some ChCs.

We crossed the *dual-tTA2* reporter to either *Unc5b-CreER; Nkx2.1-Flp* or *PV-Cre; Nkx2.1-Flp* and performed monosynaptic input tracing to ChCs and PV cells (PVCs), respectively, in three sensorimotor areas: MOs, MOp, and SSp (**Figure 7a-c**, **Figure 7 – Figure supplement 1a**). Intersecting *PV-Cre* with *Nkx2.1-Flp* is a strategy for specifically targeting PV interneurons, as *PV-Cre* also captures a sparse population of excitatory pyramidal neurons (van Brederode et al., 1991). Although PV is expressed in up to ~50% of cortical ChCs, starter cell depth distribution was found to be largely non-overlapping between injected ChCs and PVCs, likely due to the much higher abundance of PV basket cells (**Figure 7d**, **Figure 7 – Figure supplement 1d-e**).

Using serial two-photon tomography and our registration/cell detection pipeline, we mapped long-range inputs to different cortical ChC populations. Across sensorimotor cortex ChCs and PVCs integrated into highly-interconnected cortical networks. ChCs^{MOs} received ~50% of inputs from motor areas (41.0±2.3% in MOp, 4.5±0.7% in contra MOs) followed by SSp (20.4±4.2%) and SSs (9.5±0.7%) (**Figure 7e**). Long-range inputs to ChCs^{MOp} were largely dominated by sensory cortex (63.2±11.3% in SSp, 10.1±1.3% in SSs), followed by MOs (12.7±6.7%) and contra MOp (4.2±2.5%) (**Figure 7f**). In contrast, ChCs^{SSp} received only ~65% inputs total from sensorimotor (42.2±0.5%

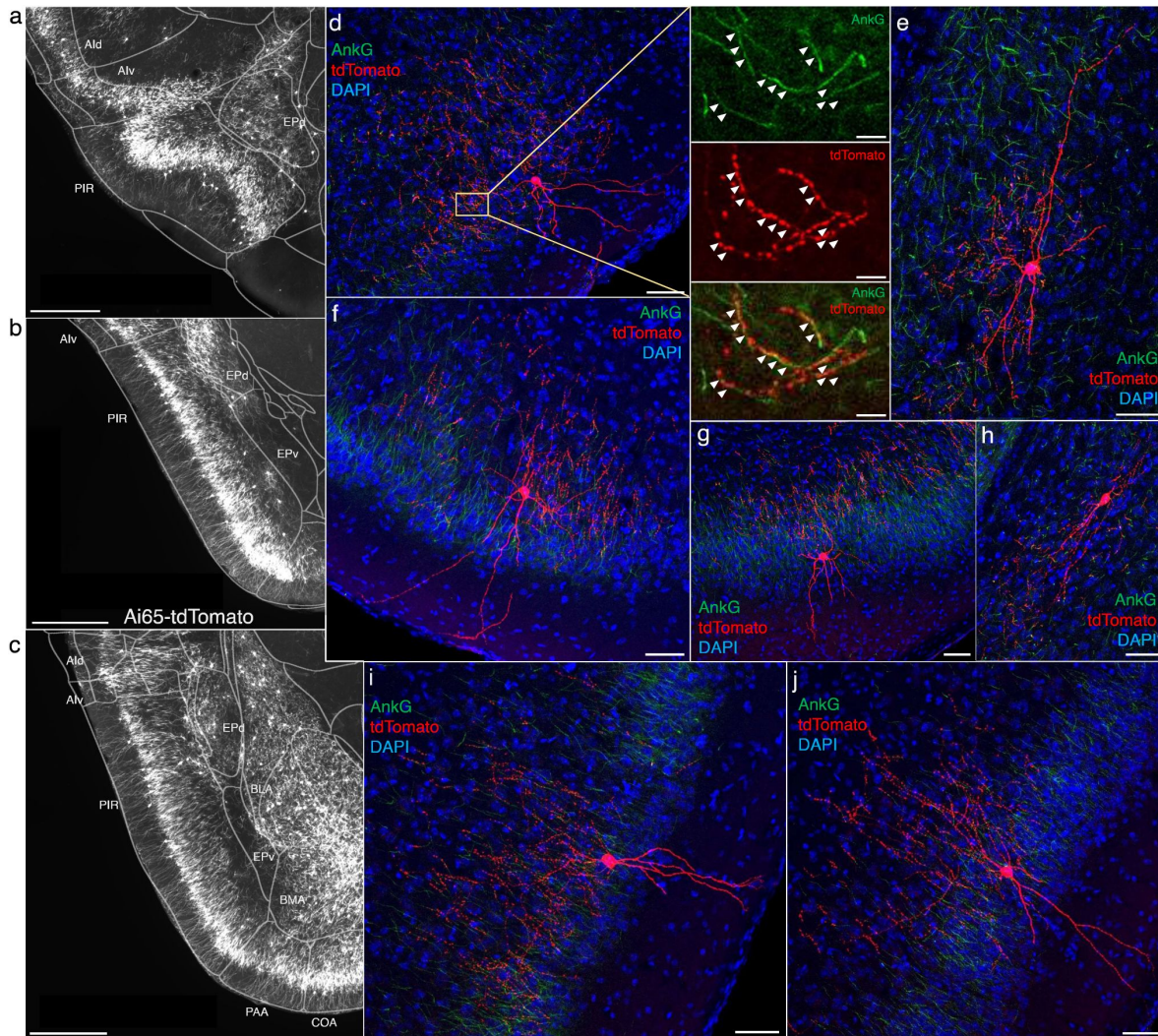


Figure 5.

AACs in piriform cortex.

(a-c) Dense AAC labelling in piriform cortex, organized from anterior (a) to posterior (c) and overlaid with ARAv3 area outlines. Note the highly laminar distribution patterns.

(d-j). Examples of single piriform AACs co-stained with AnkG (green). Note the relatively similar morphologies with only a few apical dendrites extending to the pial surface (d-g, i-j). An example of a deeper layer AAC at the border of piriform and EPv (h). White arrowheads in high-magnification insets indicate segments of overlap for RFP-labelled AAC and AnkG-labelled AIS. (Scale bars, 500µm for grayscale images, 50µm for confocal, 10µm for high-magnification insets.)

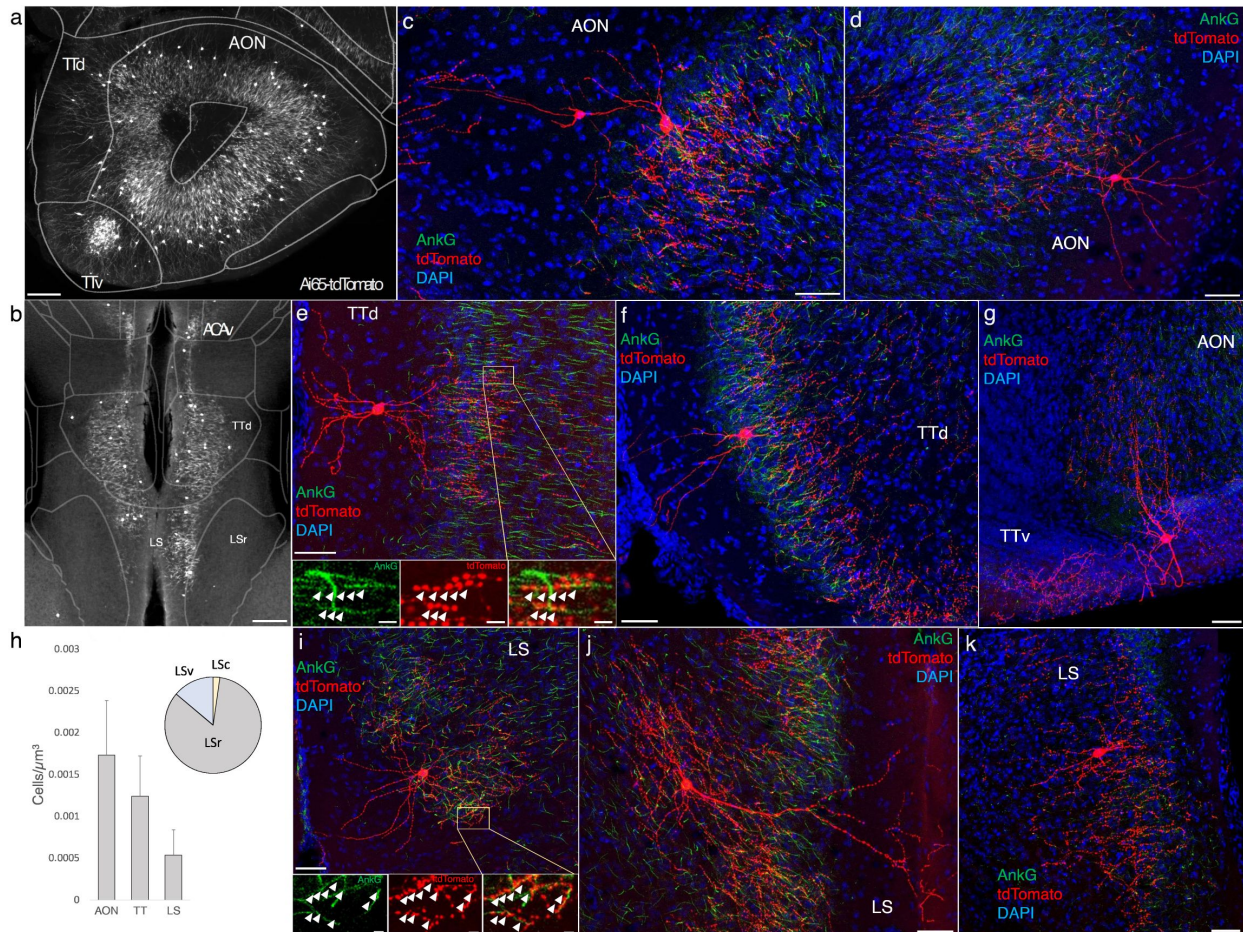


Figure 6.

Identification and distribution of novel AAC subpopulations in anterior olfactory nucleus (AON), taenia tecta (TT), and septum.

(a-b) Dense population labeling in anterior AON and TT (a) and more posterior sections containing the TT and LS (b). Anteriorly, AACs are more concentrated in ventral TT while posteriorly in dorsal TT. Within LS, AACs tend to occupy the medial most portions along the midline.

(c-g, i-k) Single cell (confocal) labeling of AACs in anterior olfactory nucleus (c-d), taenia tecta (e-g), and lateral septum (i-k). Note that despite areal variations, individual AACs preserve their stereotyped morphological characteristics (apical-oriented dendrite and basal-oriented axon arbor) that conform to laminar patterns of targets in each anatomical area. High-magnification insets: white arrowheads indicate segments of AnkG and RFP apposition. (Scale bars, 200 μm for grayscale panels, 50 μm and 5 μm for single cell examples and insets.)

(h) Normalized cell density (cells/ μm^3) for AACs in AON, TT, and lateral septum. Within lateral septum, the majority of AACs are located in rostral compartments (LSr). Data are mean $\pm$ SEM.

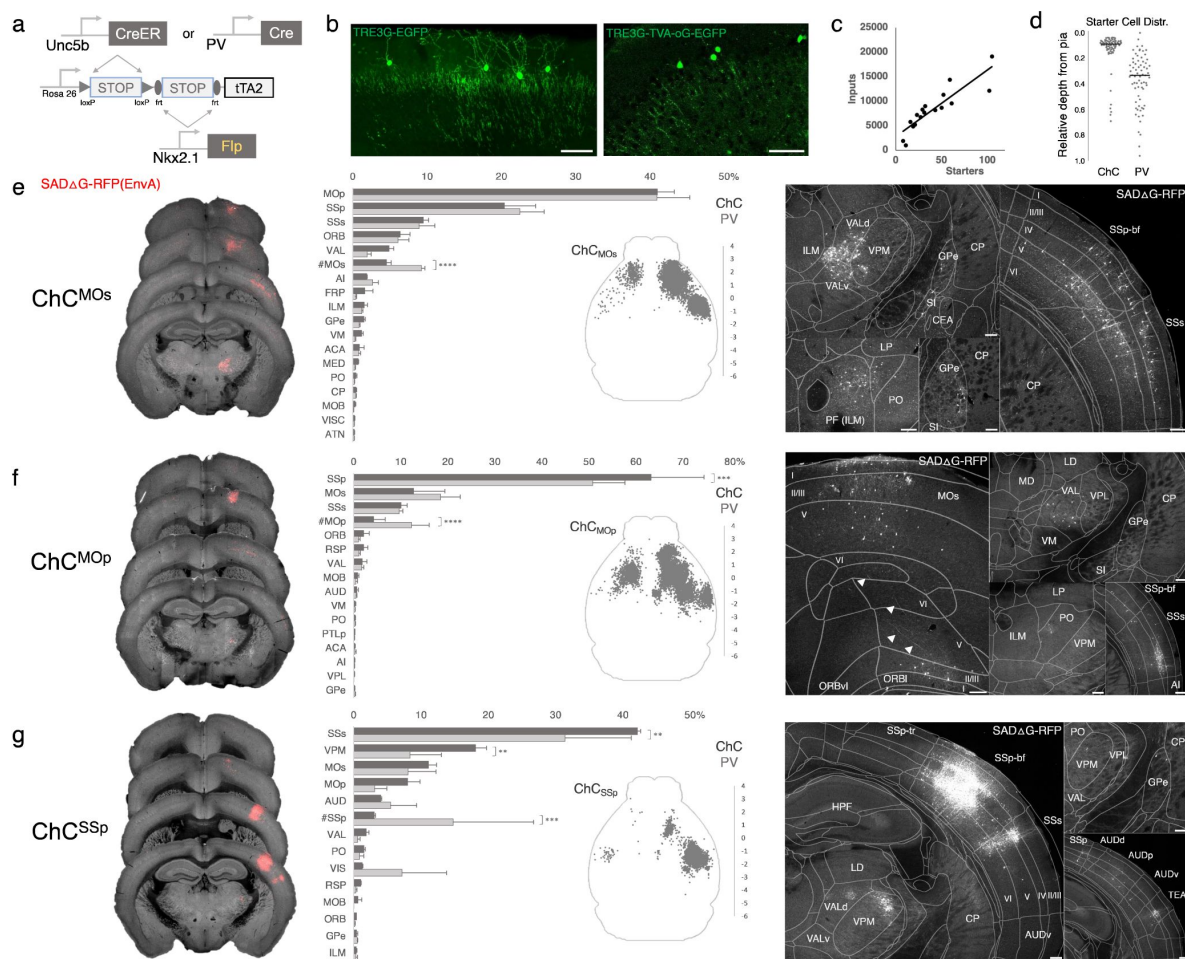


Figure 7.

Intersectional tTA2 conversion enables viral access and input tracing to ChCs in sensorimotor cortices.

(a) Schematic of intersectional tTA2-conversion strategy. Expression of *Nkx2.1-Flp* and subsequent *Unc5b-CreER* or *PV-Cre* results in activation of tTA2 expression in AACs. This provides a genetic handle for versatile viral targeting and manipulation.

(b) Cortical injection of TRE3G-promoter AAV vectors enables specific targeting of ChCs (green, left). A similar backbone was used to construct a TRE3G rabies starter AAV for monosynaptic input tracing, which contains both TVA and optimized rabies glycoprotein (right). Compared to TRE3G-EGFP, the TRE3G starter AAV has weaker EGFP labeling of neurites. (Scale bar, 200µm.)

(c) Linear regression fit of inputs cells/starter cells. Each point represents one animal, $n = 18$, $r = 0.8874$, slope = 135.51.

(d) Relative pia-to-white matter depth distribution of starter cells (expressing both AAV starter and rabies). ChC starter cell tended to cluster closer to the pia while PV cells were more broadly distributed throughout the cortical upper layers, reflecting the differences in the distribution of the two subtypes.

(e-g) Synaptic input source to ChCs in MOs, MOp, and SSp, with PV cells as comparison. Only long-range inputs (i.e. outside the ARAV3 area injected) are plotted.

Left: representative coronal sections from three indicated cortical areas overlaid with macroscale view of rabies tract labeling (in red, gray is autofluorescence) to each subpopulation of ChC.

Middle: Histogram comparing percentage of inputs from each structure innervating the indicated cortical subpopulation of ChCs (dark gray) and PV (light gray), sorted from largest input source to smallest. Inset: Representative top-down map of all inputs to AACs from a single animal.

Right: Representative sections highlighting select sources of rabies-labelled inputs to each ChC population, overlaid with ARAV3 boundaries. (Scale bars, 200µm.) For e-g, data are mean $\pm$ SEM, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ indicates $P \geq 0.05$ (two-way ANOVA with post hoc Bonferroni correction); for scatterplot in d, median is plotted. Homotypic projections to injection site from contralateral hemisphere are denoted by (#). All images showing EGFP were immunostained for signal amplification, while rabies-RFP shown in panels e-g were native fluorescence.

For each MOs, MOp and SSp, $N = 6$ mice for (3 *Unc5b; Nkx2.1*, 3 *PV; Nkx2.1*; total $N = 18$ mice)

in SSs, $11.1 \pm 1.2\%$ in MOs, $7.8 \pm 1.9\%$ in MOp, $3.03 \pm 0.2\%$ in contra SSp), with considerably more inputs from thalamus and other cortical areas (**Figure 7g**). Thalamocortical (TC) afferents to ChCs^{MOs} and ChCs^{MOp} were primarily from VAL, whereas TC inputs to ChCs^{SSp} were mostly from VPM. In addition to sensorimotor, other cortical areas such as ORB, FRP, AI, VIS, ACA, RSP, and AUD comprised a minor source of long-range inputs. Interestingly, we identified sparse inputs from GPe directly to ChCs and PV in all three cortical areas. Overall input patterns were comparable between ChCs and PV, although ChCs received significantly less homotypic inputs from the contralateral hemisphere for each injection site (**Figure 7e-g**).

Monosynaptic input tracing to AACs in CA1

The functional role of AACs is perhaps best understood in hippocampus, where CA3 AACs tend to fire at distinct epochs compared to other cell types and near the peak of theta oscillations (Viney et al., 2013). Little is known about the inputs to AACs that might drive this cell-type functional distinction, although non-specific tracers have identified an inhibitory connection from medial septum (MS) to AACs in CA3 (Viney et al., 2013). More recently, we used *Unc5b-CreER* to functionally characterize the firing patterns of AACs^{CA1} during whisking and locomotion (Dudok et al., 2021). Here, we applied cell type targeted retrograde monosynaptic tracing to map long-range inputs to AACs^{CA1} (**Figure 8a-b**).

Compared to cortical ChCs, AACs^{CA1} received inputs from more diverse sets of sources. Within the hippocampus formation, AACs^{CA1} received asymmetric inputs from each hemisphere, with relatively a higher fraction in CA3 contralaterally ($29.0 \pm 2.4\%$) but higher ipsilaterally in DG ($4.3 \pm 1.5\%$) and ENT ($11.6 \pm 3.6\%$) and SUB ($15.2 \pm 5.2\%$) (**Figure 8c**). Surprisingly, while the aforementioned study (Viney et al., 2013) found inhibitory connections from MS to AACs in CA3, we found in AACs^{CA1} over two-fold higher input connectivity from adjacent NDB ($5.1 \pm 0.9\%$) compared to MS ($2.0 \pm 0.2\%$). (**Figure 8d-e**). Immunohistochemistry analysis found the majority of these long-range inputs were inhibitory ($56.8 \pm 4.8\%$) and a sizeable proportion ChAT-positive ($27.6 \pm 4.0\%$) (**Figure 8f-j**). AACs^{CA1} also received diverse cortical inputs, including from VIS, RSP, AUD, SSp, PTLp, SSs, Tea, and PERI, as well as TC afferents from ATN (**Figure 8l-p**; see abbreviations in appendix).

Monosynaptic input tracing to AACs in basolateral amygdala

Comparatively little is known about the function or circuit role of AACs^{BLA}. Past studies have had to largely rely on post hoc identification from paired recordings, demonstrating their potent inhibitory effects (Veres et al., 2014). However, without cell type-specific tools for AACs^{BLA}, the long-range afferent connectivity that might drive these effects has remained virtually unknown. We applied our intersectional targeted approach to reveal the monosynaptic inputs to AACs^{BLA} (**Figure 9a-b**).

AACs^{BLA} received highly diverse sets of inputs (**Figure 9c**). AACs^{BLA} received strongest afferents from PIR ($13.1 \pm 1.1\%$) and the hippocampal-entorhinal network (ENT $12.7 \pm 0.2\%$, CA1 $10.5 \pm 2.0\%$, SUB $8.0 \pm 0.1\%$) (**Figure 9e**, h-i). The next strongest inputs originated from cortical amygdala areas (TR, PAA, COA) and cortical subplate nuclei (CeA, LA, EP, BMA) (**Figure 9d**). TC afferents were largely from MTN ($2.6 \pm 0.4\%$), while cortical projections were identified in AI, AUD, PERI, ECT (**Figure 9f**). Input cells in LZ and MEZ of hypothalamus were found to be glutamatergic, while those from GPe and EP were GABAergic (**Figure 9j-o**).

Discussion

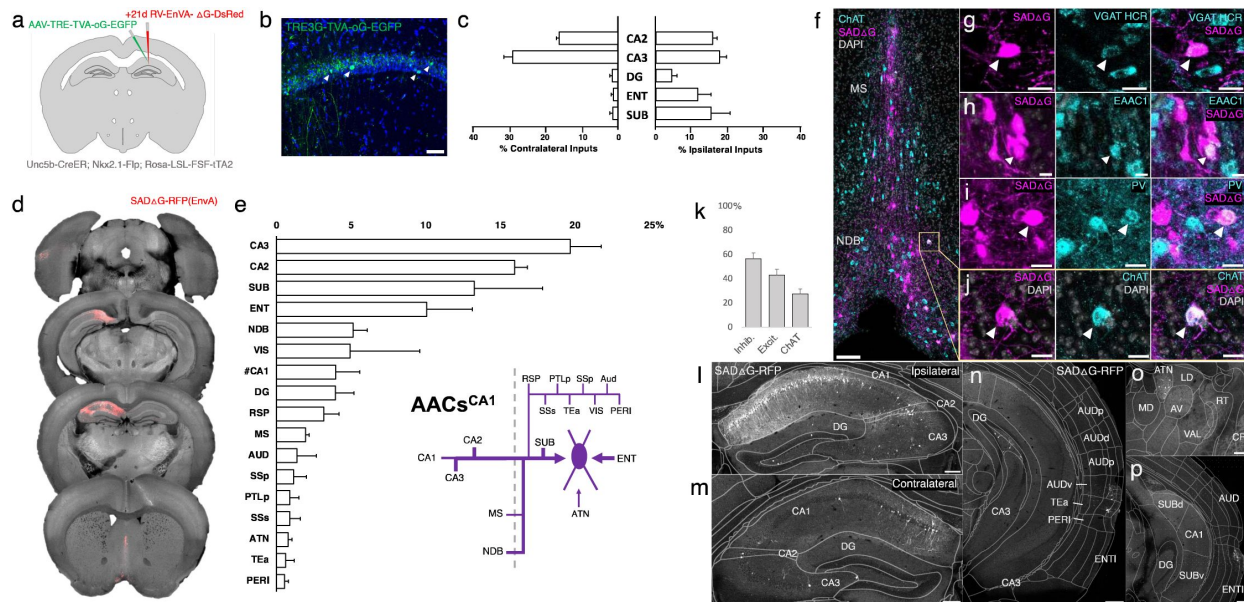


Figure 8.

Monosynaptic input tracing to AACs^{CA1} of hippocampus.

- (a) Schematic of retrograde synaptic tracing from CA1 AACs with site of viral injection.
- (b) Starter AAV expression in CA1 (green). White arrowheads show soma position is largely confined to the pyramidal cell layer. (Scale bar, 50 μ m.)
- (c) Comparative input distribution from ipsilateral (right) and contralateral (left) hippocampal compartments.
- (d) Mesoscale rabies labeling of presynaptic input cells (red) to AACs^{CA1}.
- (e) Histogram of percentage of total inputs from each structure, sorted from largest to smallest. Inset: Summary schematic of input sources to AACs^{CA1}. AACs^{CA1} receive strong innervation from entorhinal, subiculum, and contralateral hippocampus, with more minor innervation from thalamus, medial septum, diagonal band nucleus, and various cortical areas.
- (f-j) Immunohistochemistry and HCR RNA-FISH marker analysis of input cells in medial septum (MS) and nucleus of the diagonal band (NDB), indicated by color-coded labels. Examples of co-labeled cells are shown following HCR RNA-FISH for the inhibitory marker vGAT (g) and immunohistochemistry for the excitatory marker EAAC1 (h), PV (i) and ChAT (j). White arrowheads indicate co-labelled cells for each marker. (Scale bars, 50 μ m and 20 μ m for high-magnification insets.)
- (k) Quantification of marker colocalization with rabies labeling in MS and NDB.
- (l-p) Representative coronal sections highlighting select sources of rabies-labelled inputs to AACs^{CA1}, overlaid with ARAV3 atlas boundaries. Inputs within hippocampus were mostly from within CA1 both ipsilateral (l) and contralateral (m) to the injection site. Input cells were also found in adjacent cortical areas more posterior such as entorhinal cortex (ENT), auditory cortex (AUD), and temporal association area (TEa) (n-p). Thalamic inputs were largely confined to the anterior group of the dorsal thalamus (ATN) (o). (Scale bars, 200 μ m.)

For c, e, and k, data are mean $\pm$ SEM from N=4 mice. Homotypic projections to injection site from contralateral hemisphere are denoted by (#CA1) in e. EGFP in panel b and rabies-RFP in panels f-j were immunostained for signal amplification, while rabies-RFP shown in panels l-p were native fluorescence.

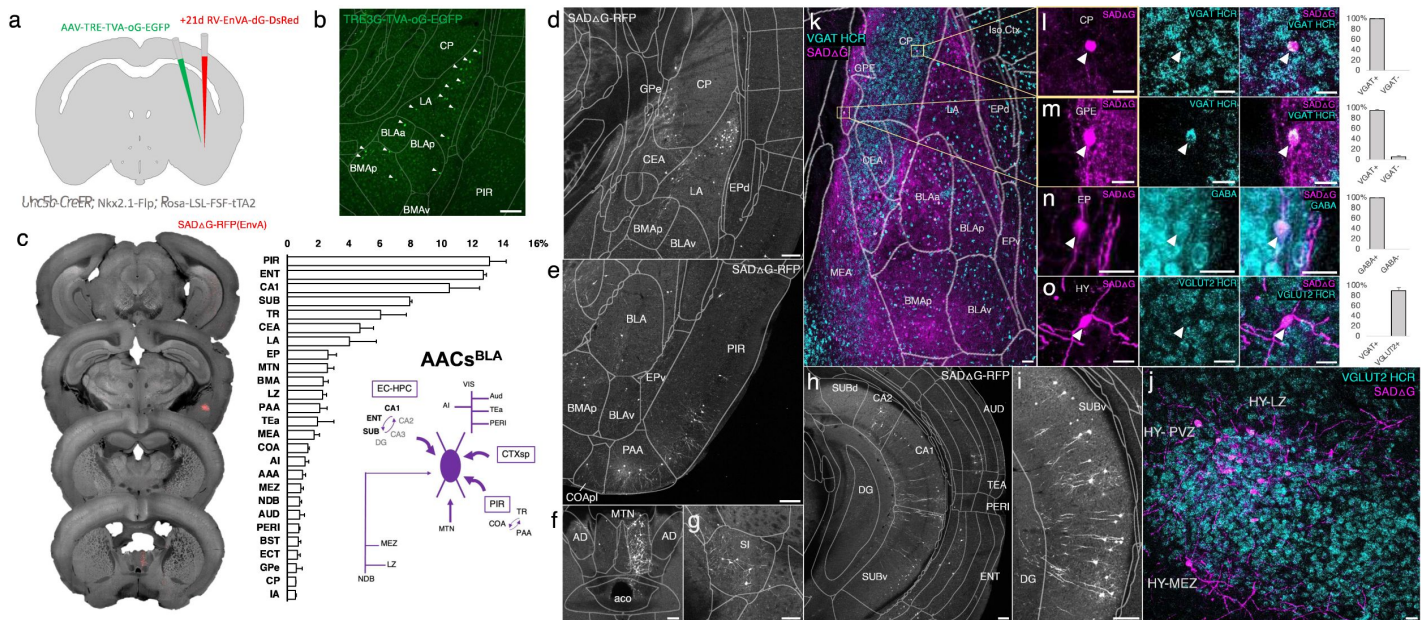


Figure 9.

Monosynaptic input tracing to AACs^{BLA}.

(a) Schematic of retrograde synaptic tracing from BLA AACs with site of viral injection.

(b) Starter AAV distribution as indicated by white arrowheads, overlaid with ARAv3 atlas. (Scale bar, 200µm.)

(c) Left: Mesoscale rabies labeling of presynaptic input cells (red) to AACs^{BLA}. Right: Histogram of percentage of total inputs from each structure, sorted from largest to smallest. Inset: Summary schematic synaptic input source to AACs^{BLA}. AACs^{BLA} are innervated by diverse sets of inputs, with strongest innervation from entorhinal cortical-hippocampal network (EC-HPC), piriform areas (PIR), cortical subplate (CTXsp), and minor subcortical and cortical inputs.

(d-j) Representative coronal sections of rabies-labelled inputs (grey) across input structures, overlaid with ARAv3 boundaries. (Scale bars, 200µm.) Inputs to AACs^{BLA} were found in amygdalar and cortical-amygdalar areas (d-e), piriform (e), midline group of the dorsal thalamus (f), substantia innominata (g), posterior hippocampus and cortex (h) and the ventral subiculum (i). Examples of dense input cells in medial (MEZ) and lateral (LZ) hypothalamus overlaid with excitatory vGLUT2 HCR RNA-FISH (j).

(k-o) Immunohistochemistry and RNA-FISH marker analysis of input cells in striatum/CP, GPe, EP, and hypothalamus. VGAT FISH or GABA IHC were used for identifying GABAergic input cells (l-n) while vGLUT2 FISH was used an excitatory marker (o) as indicated. White arrowheads indicate co-labelled cells. (Scale bars, 50µm and 20µm for high-magnification insets.) Right: Quantification of marker colocalization with rabies labeling for each corresponding area. Inputs to AACs^{BLA} from CP, GPe, and EP were inhibitory, while inputs from hypothalamus were excitatory. Data are mean ± SEM from N=3 mice.

Rabies-RFP in panels j-o were immunostained for signal amplification, while EGFP in panel b and rabies-RFP shown in panels d-i were native fluorescence.

Specific and comprehensive targeting of ground truth cell types

A major challenge in studying the functional organization of GABAergic inhibitory circuits, and neural circuits in general, is establishing specific and comprehensive genetic access to ground truth cell types. Here, leveraging an intersectional strategy that integrates lineage and molecular markers, we have achieved near-perfect specificity and near-complete comprehensiveness in targeting a bona fide interneuron type. This strategy will facilitate studying the connectivity, function, and development of AACs across brain regions, thereby providing a robust experimental platform for integrating multi-modal analysis of a ground truth cell type across brain circuits. We recognize that it is currently not possible to fully assess the “completeness” of AAC targeting; this may require a comparison with complete labeling by anatomical ground truth, such as dense EM reconstruction of all AACs across the brain volume. Another practical drawback of our approach is the requirement of combining three engineered genetic alleles. In this context, the *Unc5b-CreER* line by itself is already useful for AAV-mediated targeting of AACs (Dudok et al., 2021 [DOI](#)). Because most AAVs are neurotrophic, Cre-activated AAVs would bypass endothelial cells to target only AACs in the *Unc5b-CreER* line, even though this approach is not suited to quantify brain-wide AAC patterns. Beyond relying on germline engineering, the recently invented CellREADR technology (Qian et al., 2022 [DOI](#)) will further simplify AAC targeting by identifying an RNA sensor of *Unc5b* mRNA and deliver it with an AAV vector. As *Unc5b* is a conserved AAC marker from rodents to primates including humans, the CellREADR approach may enable genetic access to AACs across mammalian species.

Implications of brain-wide AAC distribution pattern

The initial discovery of neocortical AACs was based on their striking chandelier-like morphology (Szentágothai, 1975 [DOI](#)), reflecting the spatial arrangement of their exclusive synaptic targets – the AIS of cortical pyramidal neurons (Somogyi, 1977 [DOI](#); Somogyi et al., 1982 [DOI](#)). This defining feature of synapse specificity led to subsequent discoveries of AACs in the hippocampus and BLA. These findings have relied on meticulous efforts of skilled neuroanatomists to correctly identify AACs, mostly post hoc, by synapse specificity at AIS (Bienvenu et al., 2012 [DOI](#); Veres et al., 2014 [DOI](#)). Since then, it has been widely assumed that AACs are restricted to these brain structures, and likely to the mammalian species. Here, based on developmental lineage and key molecular markers that likely include all AACs, our results reveal a vastly expanded and likely complete brain distribution pattern of AACs. Most importantly, we found that pAACs are deployed to the entire pallium-derived brain structures (Puelles, 2017 [DOI](#)), including not only the dPAL-derived neocortex and mPAL-derived hippocampal formation, but also the IPAL-derived claustrum-insular complex, the vPAL-derived extended amygdaloid complex, piriform cortex, and other olfactory centers such as AON, taenia tecta. In addition, subregions of the lateral septum and hypothalamus also contain pAACs.

Among the pallium-derived structures, the density of AACs vary substantially within and across regions. For example, within the neocortex, ChCs are highest in perirhinal and lowest in retrosplenial cortex. Across cortical layers, AACs are highly concentrated in L2, enriched in L5/6, and significantly less abundant in middle layers. Consistent with these results, the number and density of GABAergic synapses at AIS also vary substantially among different PyNs within and across cortical layers (DeFelipe et al., 1985 [DOI](#); Fish et al., 2013 [DOI](#); Schneider-Mizell et al., 2021 [DOI](#)). Similar variations are seen across the hippocampal formation, where CA2 exhibit the highest and DG the lowest density. It is also possible that these regional differences might reflect the distinction of PN types and/or their inherent physiological and connectivity properties that might require different levels and ratio of AAC control.

In particular, the finding of a striking abundance of AACs in olfactory centers across piriform cortex, AON, and taenia tecta suggests strong inhibitory regulation of PNs at AIS in these regions. Although the functional significance is not yet clear, the simpler circuit architecture and strong

link to innate rodent behaviors of these olfactory centers may facilitate uncovering the logic of AAC function. Furthermore, as these olfactory circuits are evolutionarily preserved across vertebrate species (Zeppilli et al., 2023 [↗](#)), our finding raises the possibility that AACs might be present in the CNS of other vertebrates, beyond the mammalian brain.

Notably, although the MeA and BST comprise mostly GABAergic neurons that are primarily generated from the subpallium, they contain subsets of glutamatergic projection neuron that migrate from pallium origin (Beyeler & Dabrowska, 2020 [↗](#)) or from the third ventricle (Garcia-Moreno et al., 2010 [↗](#)). The true AAC identity in these regions, and in several other regions such as the hypothalamus, remains to be validated by synapse innervation at AIS of GLU PNs. Nevertheless, based on current results, our overarching hypothesis is that AACs are deployed across all pallium-derived structures to control the AIS of GLU PNs, including those in MeA and BST. Our results also indicate that AACs are absent from the rest of the CNS. This finding suggests a major distinction between the physiology and functional organization of pallium-derived GLU PNs versus those in other CNS regions.

AACs as a model for understanding neuronal cell type organization across regions and species

The rapid scaling of single cell RNA sequencing enables the generation of massive transcriptome datasets across brain regions and species (Trygve E. Bakken et al., 2021 [↗](#); Hain et al., 2022 [↗](#)). These datasets provide unprecedented opportunities to discover the organization and relationship of cell types within and across brain regions, which reflect their gene regulatory programs and developmental origin. Cross species analyses promise to further reveal the evolutionary conservation and divergence of brain cell types (T. E. Bakken et al., 2021 [↗](#); Tosches, 2021 [↗](#)). However, transcriptomic cell homologies between brain regions and species, especially more distantly related regions and species, are often discernible only at a relatively coarse level and do not fully capture the biological complexity (Tosches, 2021 [↗](#)). A major challenge is to evaluate statistically defined transcriptomic similarity against biological ground truth, such as anatomic connectivity and developmental relationship. In this context, the most appealing attribute of AACs is their singularly defining synapse specificity at AIS of GLU PNs, which can be readily measured and validated. Under this overarching connectivity-based definition, all other molecular and phenotypic variations can be assessed and organized accordingly regardless of brain regions and species. Therefore, genetic access to AACs across brain regions provides a rare and compelling ground truth platform that can unambiguously identify this cell type across regions and species. This will further facilitate transcriptomic and epigenomic analysis to discover the gene regulatory program of AACs and contribute to understanding the principles of cell type organization by linking developmental genetic program, circuit connectivity and function, and evolutionary trajectory.

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Author Contributions

Z.J.H. and B.L. conceived the study and obtained funding. Z.J.H. coordinated the study. Z.J.H., B.L., and R.R. designed the experiments. R.R., G. K., S.K., A.P., J.H., and S.Z. conducted mouse breeding, anatomy, immunohistochemistry, imaging and quantification. R.R. and W.G. performed virus injection experiments. R.R. and J.H. conducted whole-brain STP tomography and analysis of cell distribution patterns. S.Z. performed HCR ISH experiments. R.R. and Y.Q. generated the starter AAV virus. Z.J.H. and R.R. wrote the manuscript with contributions and edits from B.L. and S.K.

Materials and Methods

Key Resources Tables

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Antibody	Anti-AnkryinG (mouse monoclonal)	Neuromab	Cat#: 75-146	1:500
Antibody	Anti-ChAT (goat polyclonal)	Millipore	Cat#: AB144P	1:300
Antibody	Anti-EAAC1 (goat polyclonal)	Millipore	Cat#: AB1520	1:500
Antibody	Anti-GABA (rabbit polyclonal)	Sigma	Cat#: A2052	1:500
Antibody	Anti-GFP (chicken polyclonal)	Aves Labs	Cat#: 1020	1:1000
Antibody	Anti-parvalbumin (mouse monoclonal)	Sigma	Cat#: P3088	1:1000
Antibody	Anti-Phospho-IκBα (rabbit polyclonal)	Cell Signaling Technology	Cat#: 2859	1:500
Antibody	Anti-RFP (rabbit polyclonal)	Rockland	Cat#: 600-401-379	1:1000
Commercial assay or kit	HCR v3.0	Molecular Instruments		
Other	AAVDJ-TRE3g-EGFP	Vigene		Adeno-associated virus, titer: 5×10^{12} genome copies (gc)/ml
Other	AAVDJ-TRE3g-TVA-oG-EGFP	Vigene		Adeno-associated virus, titer: 9.24×10^{12} genome copies (gc)/ml
Other	G-Deleted Rabies dsRedXpress	Salk Institute		Rabies virus, titer: 5.0×10^7 colony-forming units (cfu)/ml
Software, algorithm	ImageJ	NIH	RRID:SCR_003070	
Software, algorithm	MATLAB	Mathworks	R2017b RRID:SCR_001622	
Strain (Mus musculus)	Ai65	The Jackson Laboratory	021875 RRID:IMSR_JAX:021875	
Strain (Mus musculus)	C57BL/6J	The Jackson Laboratory	000664 RRID:IMSR_JAX:000664	

Strain (Mus musculus)	dual-SynaptotagminEGFP-2A-tdTomato	The Jackson Laboratory	030206 RRID:IMSR_JAX:030206	
Strain (Mus musculus)	dual-tTA2	The Jackson Laboratory	036304 RRID:IMSR_JAX:036304	
Strain (Mus musculus)	Nkx2.1-Cre	The Jackson Laboratory	008661 RRID:IMSR_JAX:008661	
Strain (Mus musculus)	Nkx2.1-FlpO	The Jackson Laboratory	028577 RRID:IMSR_JAX:028577	
Strain (Mus musculus)	Pthlh-FlpO			
Strain (Mus musculus)	PV-Cre	The Jackson Laboratory	017320 RRID:IMSR_JAX:017320	
Strain (Mus musculus)	Unc5b-CreER			

Animals

All experiments were carried out in accordance with the guidelines of the Animal Care and Use Committee of Cold Spring Harbor Laboratory (CSHL). Mice were housed in groups of five per cage or singly if pregnant under standard vivarium conditions with a 12 h light/dark cycle. Unc5b-CreER (Dudok et al., 2021 [DOI](#)) or PV-Cre (JAX, Stock 017320) were crossed to Nkx2.1-Flp (JAX, Stock 028577). Pthlh-Flp (unpublished) was crossed to Nkx2.1-Cre (JAX, Stock 008661). For each Cre/Flp combination, offspring hemizygous for both alleles were crossed to Ai65 (JAX, Stock 021875), Rosa26-loxpSTOPloxp-frtSTOPfrt-synaptotagminEGFP-2A-tdTomato (JAX, Stock 030206), or Rosa26-loxpSTOPloxp-frtSTOPfrt-tTA2 (JAX, Stock 036304) reporter mice. Mice with Unc5b-CreER were induced by tamoxifen i.p. (TM; Sigma-Aldrich, Cat# T5648) administered either 1) for dense labelling and input tracing, 100mg/kg for five consecutive days starting at P55-60 2) for single cell labeling, 25mg/kg for 1-2 consecutive days at P55-P60. The dosage and regimen of tamoxifen for dense AAC labelling was the maximum based on established protocols for conditional knockouts and one that was well-tolerated. For single cell labeling, the tamoxifen dosage was determined empirically for reliable labeling of non-overlapping AACs in different brain areas. Animals were fed ad libitum and their health status were routinely monitored. Both male and female animals were used in all experiments, with 3-4 mice total for each injection site x genotype and for cell distribution analysis. Genotyping was performed using PCR and according to supplier protocols.

Immunohistochemistry

Mice were anesthetized with Avertin and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1M phosphate buffer. Following overnight post-fixation, brains were rinsed in PBS and sectioned at 50-65µm thickness with a Leica VT1000S vibratome. For immunohistochemistry, sections were first treated with a secondary-matched blocking solution (10% normal goat or donkey serum and 0.2% Triton-X100 in PBS) for 1 hr, then incubated overnight at 4°C with primary antibodies diluted in 5% blocking solution. Sections were washed three times in PBS and incubated for 2h at room temperature with corresponding secondary antibodies, Goat or Donkey Alexa Fluor 488, 568 or 647 (1:500, Life Technologies) and DAPI to label nuclei (1:1000 in PBS, Life Technologies, Cat# 33342). Sections were washed three times with PBS and dry-mounted on slides using Fluoromount-G (SouthernBiotech, Cat# 0100-01) mounting medium. Primary antibodies used were Anti-AnkryinG (1:500, Neuromab, Cat#: 75-146), Anti-ChAT (1:300, Millipore, Cat#: AB144P), Anti-EAAC1 (1:500, Millipore, Cat#: AB1520), Anti-GABA (1:500,

Sigma-Aldrich, Cat#: A2052), Anti-GFP (1:1000, Aves Labs, Cat#: 1020), Anti-parvalbumin (1:1000, Sigma-Aldrich, Cat#: P3088), Anti-Phospho-IkBa (1:500, Cell Signaling Technology, Cat#: 2859), Anti-RFP (1:1000, Rockland, Cat#: 600-401-379). Samples for AAC validation were collected from 6 mice given 25mg/kg for 1-2 consecutive days, as described above. Validation was conducted with high-magnification confocal microscopy and defined by a cell exhibiting at least two RFP-labelled axons colocalized with AIS labelled by AnkryinG or Phospho-IkBa.

Floating section two-color in situ hybridization

In situ hybridization was performed using hybridization chain reaction (HCR v3.0, Molecular Instruments). Samples post-fixed in 4% PFA at 4°C were cryoprotected in 30% sucrose solution in RNase-free PBS (DEPC-PBS) at 4°C for 48 hr, frozen in Tissue-Tek O.C.T. Compound (Sakura), and stored at -80°C until sectioning. 50 µm thick coronal floating sections were collected into a sterile 24-well plate in DEPC-PBS, fixed again briefly for 5 min in 4% PFA, then placed in 70% EtOH in DEPC-PBS overnight. Sections were rinsed in DEPC-PBS, incubated for 45 min in 5% SDS in DEPC-PBS, rinsed and incubated in 2x SSCT, pre-incubated in HCR hybridization buffer at 37°C, and then placed in HCR hybridization buffer containing RNA probes overnight at 37°C. The next day, sections were rinsed 4 × 15 min at 37°C in HCR probe wash buffer, rinsed with 2X SSCT, pre-incubated with HCR amplification buffer, then incubated in HCR amplification buffer containing HCR amplifiers at room temperature for ~24 hr. On the final day, sections were rinsed in 2x SSCT, rinsed again with 2x SSCT, then mounted on slides and coverslipped with Fluoromount-G (Southern Biotech).

Microcopy and image analysis

Confocal imaging was performed on a Zeiss LSM 780 (CSHL St. Giles Advanced Microscopy Center) using 10x, 20x, or 63x objectives. Imaging for grayscale overview images was performed on a Zeiss Axioimager M2 System equipped with MBF Neurolucida Software (MBF) using 5x, 10x, or 20x objectives. Quantification and image analysis was performed using Image J/FIJI software. Statistics and plotting of graphs were done using GraphPad Prism 7 and Microsoft Excel 2010. All images shown in figures were acquired by one-photon microscopy (confocal, epifluorescence) with the exception of **Figure 1** [Supplement Figure 1](#) panels g-n, **Figure 7** [panels e-g](#), **Figure 8** [panels l-p](#), and **Figure 9** [panels d-i](#), which were acquired by serial two-photon tomography.

Viruses

To construct AAVDJ-TRE3g-TVA-oG-eGFP, TVA, the optimized rabies glycoprotein oG (Kim et al., 2016), and eGFP were linked by ‘self-cleaving’ 2A and P2A peptides and cloned into pAAV-TRE3G (Sacha Nelson). For AAVDJ-TRE3g-eGFP only eGFP was cloned. AAVs serotype DJ were packaged by a commercial vector core facility (Vigene). Genomic titers of AAVDJ-TRE3g-TVA-oG-eGFP and AAVDJ-TRE3g-eGFP were 9.24×10^{12} gc/ml and 5.0×10^{12} gc/ml, respectively. TVA, oG, and eGFP were subcloned from AAV-hSyn-FLEX-TVA-P2A-EGFP-2A-oG (Addgene, Cat# 85225). AAVs were diluted 1:5 in 1x PBS immediately prior to use. EnvA-pseudotyped G-Deleted Rabies dsRedXpress was purchased from Salk Institute (5.0×10^7 titer units (TU)/ml).

Stereotactic viral injections

Adult mice were anaesthetized with 2% isoflurane delivered by constant air flow (0.4 L/min). Analgesics ketoprofen (5 mg/kg) and dexamethasone (0.5 mg/kg) were administered subcutaneously just prior to surgery, while lidocaine (2-4 mg/kg) was applied intra-incisionally. Mice were mounted on a stereotaxic headframe (Kopf Instruments, 940 series or Leica Biosystems, Angle Two) and stereotactic coordinates identified as follows, (MOs): 2.25mm anterior to bregma, 1.2mm lateral, 0.3mm in depth; (MOp): 0.5mm anterior to bregma, 1.6mm lateral, 0.3mm in depth; (SSp): 1.12mm posterior to bregma, 3.25mm lateral, 0.3mm in depth; (CA1): 1.94mm posterior to bregma, 1.4mm lateral, 1.4mm in depth; (BLA): 1.9mm posterior to bregma, 3.38mm lateral, 4.76mm in depth. An incision was made over the scalp, a small burr hole drilled in the skull and

brain surface exposed. A pulled glass pipette with a tip of 20–30 μm containing virus was lowered into the brain. Virus was delivered at a rate of 30nL/min using a Picospritzer (General Valve Corp) for a total volume of 100–150nL for starter AAV and 300–400nL for rabies virus. The pipette remained in place for 10 min prior to retraction to prevent backflow, after which the incision was closed with 5/0 nylon suture thread (Ethilon Nylon Suture Ethicon) or Tissueglue (3M Vetbond). Mice were kept warm on a heating pad until complete recovery. For input tracing experiments, *Unc5b-CreER*; *Nkx2.1-Flp*; *dual-tTA2* (tamoxifen-induced as described above) and *PV-Cre*; *Nkx2.1-Flp*; *dual-tTA2* were injected at respective coordinates with AAVDJ-TRE3g-TVA-oG-eGFP at P75 $\pm$ 2. After a 2–3 week incubation period, a second injection at the same coordinates was performed using EnvA-pseudotyped G-Deleted Rabies-dsRedXpress. Following another 7 day incubation mice were perfused as described above.

Whole-brain STP tomography and image analysis

We used the whole-brain STP tomography pipeline described previously (Ragan et al., 2012; Kim et al., 2017; Matho et al., 2021). Perfused and post-fixed brains were embedded in 4% oxidized-agarose in 0.05 M PB, cross-linked in 0.2% sodium borohydride solution (in 0.05 M sodium borate buffer, pH 9.0–9.5). The entire brain was imaged coronally with a 20 $\times$ Olympus XLUMPLFLN20XW lens (NA 1.0) on a TissueCyte 1000 (Tissuevision) with a Chameleon Ultrafast-2 Ti:Sapphire laser (Coherent). EGFP/EYFP or tdTomato/dsRedXpress signals were excited at 910 nm or 920 nm, respectively. Whole-brain image sets were acquired as series of 12 (x) $\times$ 16 (y) tiles with 1 μm $\times$ 1 μm sampling for 230–270 z sections with a 50- μm z-step size. Images were collected by two PMTs (PMT, Hamamatsu, R3896), for signal and autofluorescent background, using a 560-nm dichroic mirror (Chroma, T560LPXR) and band-pass filters (Semrock FF01-680/SP-25). The image tiles were corrected to remove illumination artifacts along the edges and stitched as a grid sequence. Image processing was completed using ImageJ/FIJI and Adobe/Photoshop software with linear level and nonlinear curve adjustments applied only to entire images. Registration of brain-wide datasets to the Allen reference Common Coordinate Framework version 3 (CCFv3) was performed by 3D affine registration followed by a 3D B-spline registration using Elastix software (Klein et al., 2010), according to established parameters. Somata labelled by Ai65-tdtomato or rabies-dsRedXpress and cartridges labelled by synaptotagmin-EGFP were automatically detected by a convolutional network trained as described previously (Ragan et al., 2012; Kim et al., 2017; Matho et al., 2021). For AAC laminar depth, somata and cartridge coordinates were overlaid on a mask for relative cortical depth, as described. For area cell density and cortical depth analysis, we registered each dataset, reporting cells detected in each brain structure without warping the imaging channel. For rabies input tracing experiments, only inputs originating outside the ARAV3 area injected were plotted (i.e. only long-range inputs). All cell density quantifications are reported as mean cells per cubic $\mu\text{m} \pm$ SEM from n=4 mice.

Data availability

The data that support the findings of this study are presented in Source data 1.

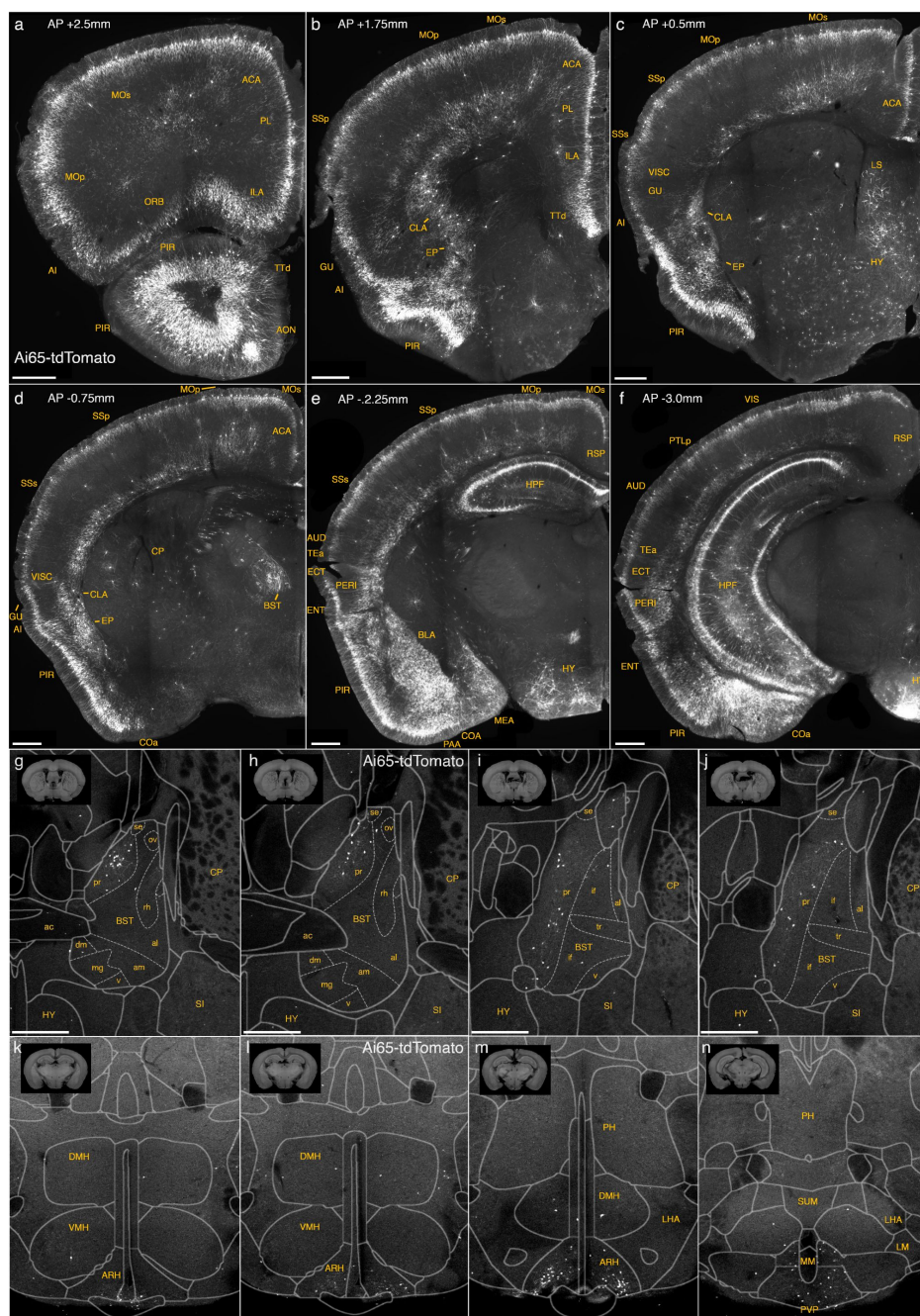


Figure 1 Supplement Figure 1.

Dense labelling of AACs across brain regions in a *Unc5b-CreER;Nkx2.1-Flp;Ai65* mouse.

(a-f) Additional representative coronal sections showing dense Ai65 expression patterns at specified anterior-posterior coordinates (from Bregma). Note labeling in cerebral cortex (a-f); hippocampus (e-f); olfactory centers, including piriform, anterior olfactory nucleus, and tenia tecta dorsal (PIR, AON, TTd) (a-b); agranular insula, claustrum, and endopiriform nucleus (AI, CLA, EP) (b-c); amygdaloid complex and extended amygdala (COA, BLA, MeA) (d-f); Bed nuclei of the stria terminalis (BST) (d); and hypothalamus (HYP) (e-f). (Scale bars, 500µm.)

(g-j) Ai65-labelled pAACs in the bed nuclei of the stria terminalis (BST) are largely confined to the principal nucleus (BSTpr), with pAACs clustering more densely in anterior BSTpr (g-h) compared to more posterior segments (i-j).

(k-n) Ai65-labelled pAACs in the hypothalamus are densest in the ventral-medially area of the arcuate nucleus (ARH), with highest density in caudal portions (k-m) and more posteriorly in the paraventricular hypothalamic nuclei (PVP) (n). pAACs are also present sparsely in the dorsal-medial nucleus of the hypothalamus (DMH) (k-m).

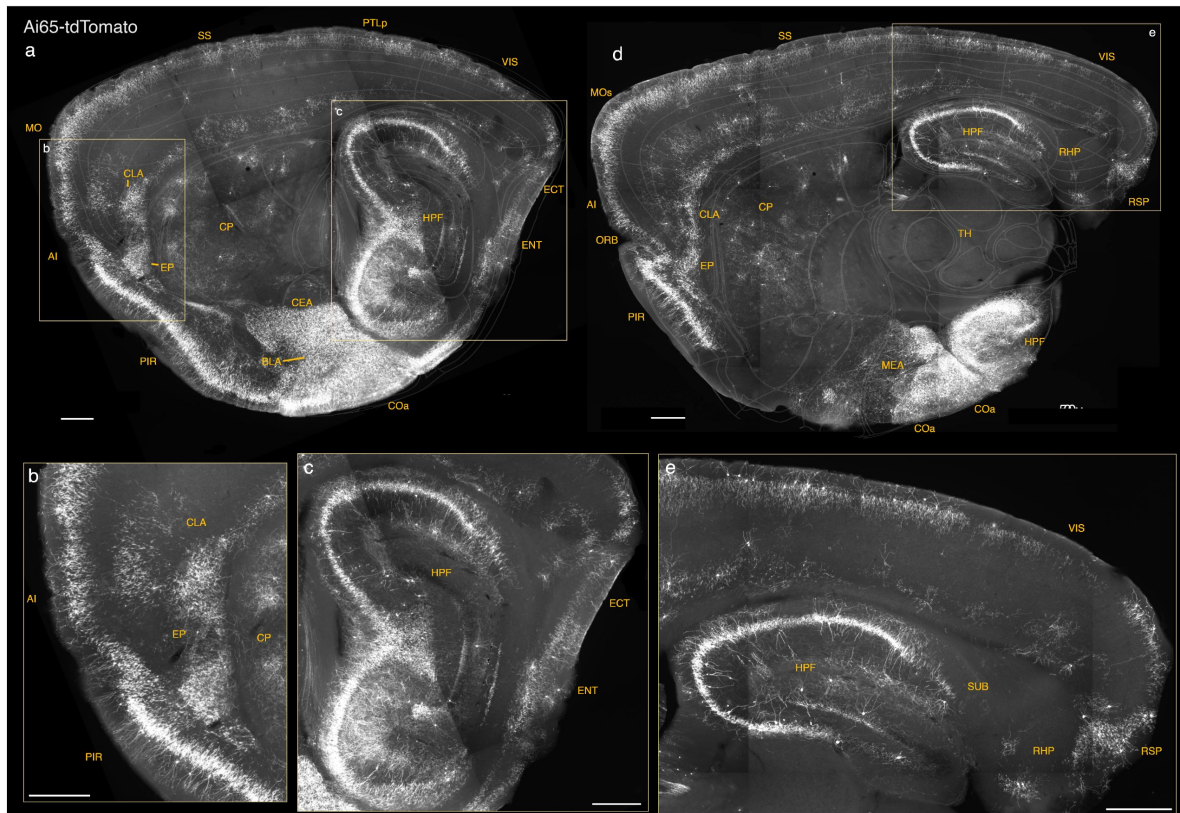


Figure 1 Supplement Figure 2.

Additional sagittal sections of *Unc5b-CreER; Nkx2.1-Flp; Ai65* mice highlighting dense pAACs in medial, lateral, and ventral pallium-derived structures.

(a-c) A lateral sagittal section showing the IPAL-derived anterior insular (AI), claustrum (CLA), endopiriform (EP) (magnified in b) and mPAL-derived hippocampal formation (magnified in c). Note the single-banded orientation of hippocampal (c) and insular (b) AACs that is absent in more inferior IPAL structures (b).

(d-e), A more medial sagittal section showing dorsal and ventral hippocampus and retrohippocampal region (RHP) (d), retrohippocampus, and visual and retrosplenial cortex (e). Compare the dual-banded AAC distribution in upper and deeper cortical layers to the single-banded pattern in hippocampus. Also note dense labeling in amygdaloid complex and extended amygdala (BLA, COa, and MeA) (a, d). (Scale bars, 500µm.)

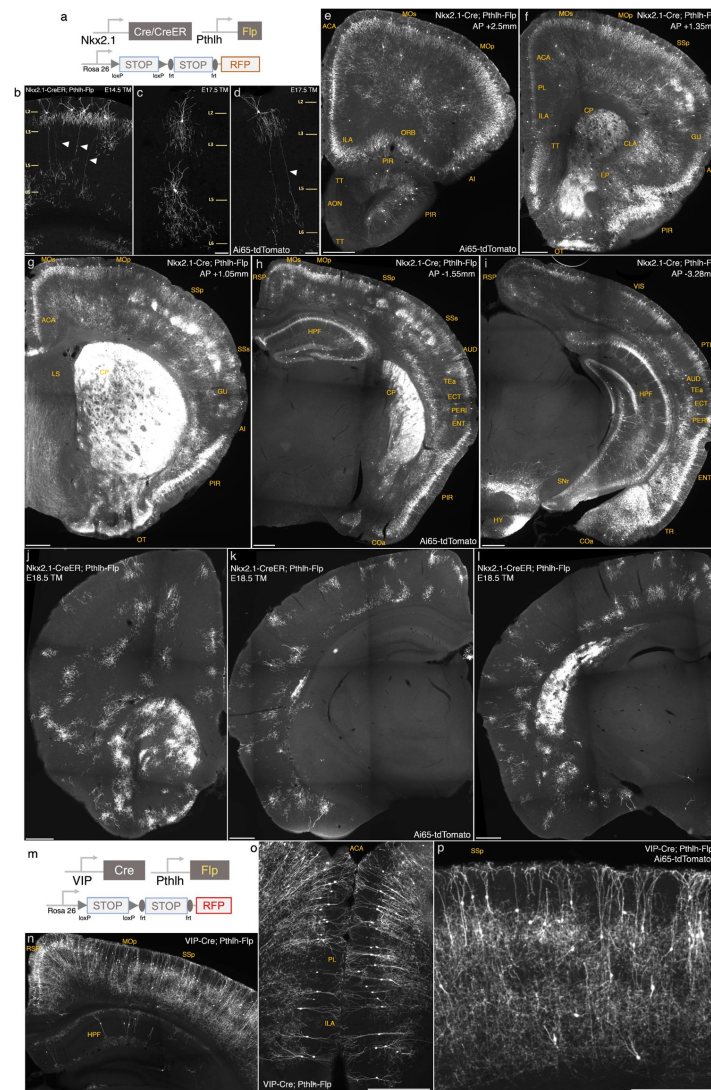


Figure 1 Supplement Figure 3.

Intersectional targeting with *Pthlh-Flp* and *Nkx2.1-Cre*, *Nkx2.1-CreER* or *VIP-Cre* to label specific subsets of AACs.

(a) Schematic of intersectional strategy.

(b-d) Tamoxifen induction of *Nkx2.1-CreER; Pthlh-Flp; Ai65* at E14.5 or E17.5 sparsely labels ChCs with diverse morphology in sensorimotor cortices. Arrowheads highlight a translaminal AAC type in layer 2, which extends axon arbors into deep layers. (Scale bars, 65µm.).

(e-i) Coronal hemisections from *Nkx2.1-Cre; Pthlh-Flp; Ai65* at specified anterior-posterior coordinates (mm from Bregma) showing pattern of dense labeling. Note labeling in cerebral cortex (e-i); hippocampus (h-i); olfactory centers, including piriform, anterior olfactory nucleus, and tenia tecta dorsal (e-h); agranular insula, claustrum, and endopiriform nucleus (AI, CLA, EP) (f); amygdaloid complex and extended amygdala (COA, BLA, MeA) (h-i); and hypothalamus (HYP) (i). Labeling by *Nkx2.1-Cre; Pthlh-Flp* is sparser in AON and anterior Ttd and absent in BST compared to *Unc5b-CreER; Nkx2.1-Flp* (Fig1). Note non-AAC labeling of layer 4 interneurons in somatosensory cortex (g-h) and striatum (CP, f-h). The absence of projection fibers in SNr (i) suggests these striatal cells are not medium spiny neurons and likely striatal interneurons (Scale bars, 500µm.).

(j-l) Coronal hemisections from *Nkx2.1-CreER; Pthlh-Flp; Ai65* following a single tamoxifen dose at embryonic day 18.5 (E18.5) shows exclusive specificity for individual ChCs across neocortex (Scale bars, 500µm.)

(m) Intersectional strategy of *Pthlh* and *VIP* using *VIP-Cre; Pthlh-Flp; Ai65* mice.

(n-p) Dense labeling of cortical and hippocampal interneurons with predominantly bipolar morphology (putative interneuron-selective cells) in cortical layers II/III and deep layers (Scale bars, 500µm.)

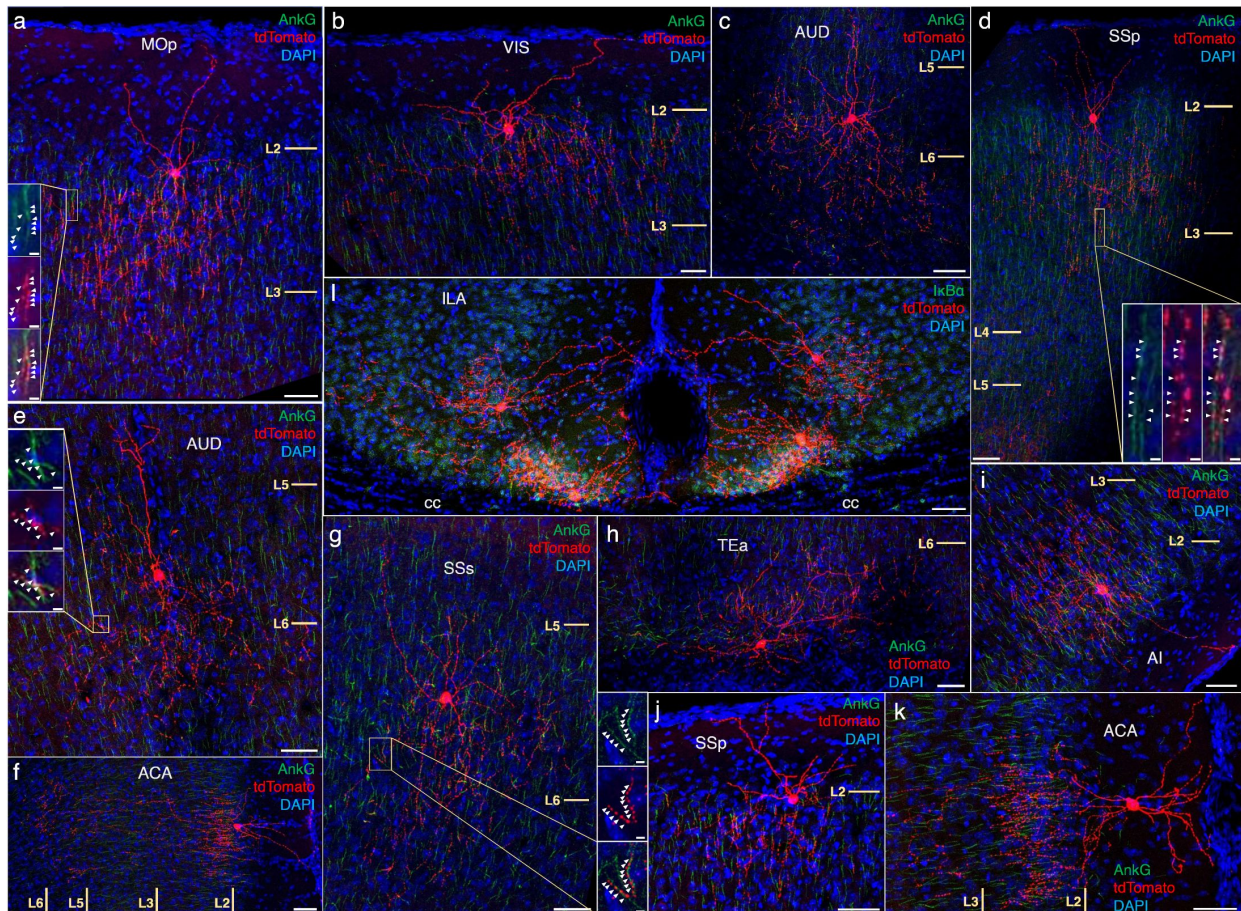


Figure 2 Supplement Figure 1.

Anatomical diversity of ChCs across neocortical areas.

AnkyrinG or IkB α immunohistochemical labeling (green) of PyN AIS was used for validation of axo-axonic targeting. Examples are shown of characteristic upper L2 ChCs in motor (a), sensory (b, d, j), cingulate (f, k), and insular cortex (i). Examples of deeper L5 or L6 ChCs in somatosensory (c, e, g) and temporal association area (TEA) (h). Note the clustering of a distinct morphological subtypes in ventromedial infralimbic cortex (l). High-magnification inset (g): white arrowheads indicate segments of overlap between AnkG and RFP. Scale bars, 50µm and 5µm for high-magnification insets.

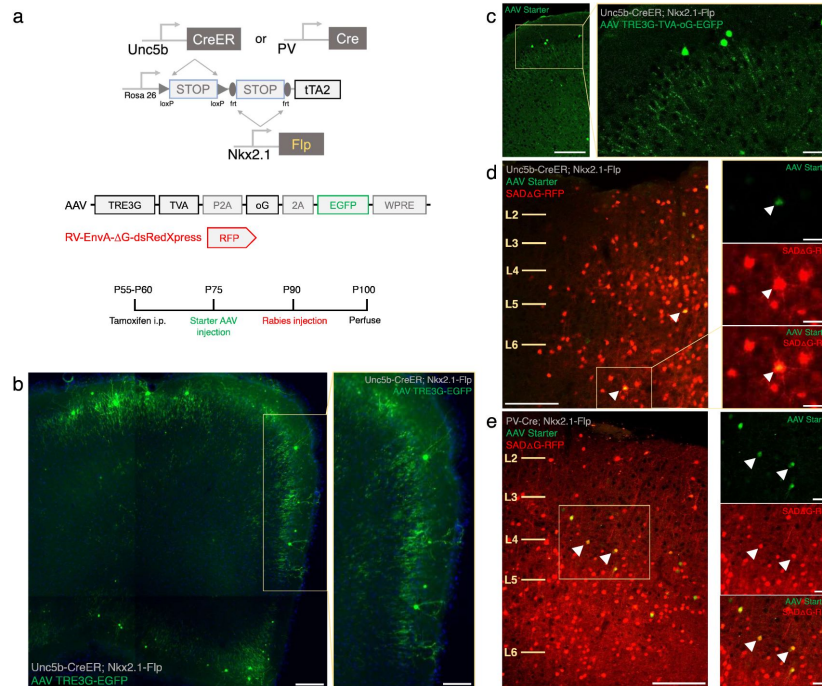


Figure 7 Supplement Figure 1.

Intersectional tTA2 conversion enables viral access and input tracing to ChCs and PV cells.

(a) Schematic and procedure for synaptic input tracing from ChC and PV interneurons. Top, driver and reporter lines to achieve tTA expression in ChC or PV cells. Middle, schematic of AAV and EnvA pseudo-typed rabies viral vectors. Bottom, timeline of tamoxifen and viral vector for retrograde synaptic tracing.

(b) Bulk injection of TRE3G-EGFP in *Unc5b-CreER; Nkx2.1-Flp; LSL-FSF-tTA2* results in efficient and specific labeling of ChCs, identifiable by laminar position and morphological features (inset). (Scale bars, 200μm and 100μm for high-magnification inset.)

(c) Starter AAV expression is comparable to TRE3G-EGFP with high specificity for ChCs in *Unc5b; Nkx2.1*, albeit with weaker EGFP neurite expression. (Scale bars, 100μm and 20μm for high-magnification insets.)

(d, e) Example of starter cells (arrowheads) co-expressing EGFP (AAV vector) and RFP (rabies vector) in *Unc5b/Nkx2.1* (d) and *PV/Nkx2.1* (e) intersections. (Scale bars, 100μm and 20μm for high-magnification insets.) All images showing EGFP were immunostained for signal amplification, while rabies-RFP shown in panels d-e were native fluorescence.

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Reviewer #2 (Public Review):

Summary:

The goals of this study were to develop a genetic approach that would specifically and comprehensively target axo-axonic cells (AACs) throughout the brain and then to describe the patterns and characteristics of the targeted AACs in multiple, selected brain regions. The investigators have been successful in providing the most complete description of the regional distribution of putative (pAACs) throughout the brain to date. The supporting evidence is convincing, and the findings should serve as a guide for more detailed studies of AACs within each brain region and lead to new insights into their connectivity and functional organization of this important group of GABAergic interneurons.

Strengths:

The study has numerous strengths. A major strength is the development of a unique intersectional genetic strategy that uses cell lineage (Nkx2.1) and molecular (Unc5b or Pthlh) markers to identify AACs specifically and, apparently, nearly completely throughout the mouse brain. While AACs have been described previously in the cerebral cortex, hippocampus and amygdala, there has been no specific genetic marker that selectively identifies all AACs in these regions.

Importantly, the current genetic strategy labels pAACs in additional brain regions, including the claustrum-insular complex, extended amygdala, and several olfactory centers in which AACs have not been previously recognized. In general, the findings provide support for the specificity of the methods for targeting AACs and include several examples of labeling near markers of axon initial segments, providing validation of their AAC identity.

The descriptions and numerous low magnification images of the brain provide a roadmap for subsequent, detailed studies of AACs in numerous brain regions. The overview and summaries of the findings in the Abstract, Introduction and Discussion are particularly clear and helpful in placing the extensive regional descriptions of AACs in context.

Weaknesses:

Considering the unique and striking characteristics of AACs, it would have been ideal to include a clear, high resolution confocal image of an AAC from the Unc5b;Nkx2.1 mouse that would display the beauty of these cells with their numerous cartridges of axon terminals, emanating from a single AAC. While several cells are illustrated, the processes are often obscured by other labeling or the background created by the blue Dapi labeling. A high-resolution image of an isolated cell would not only support the identity of the cells as AACs but also demonstrate the potential advantages of their labeling for more detailed anatomical and neurophysiological studies. High magnification views of the axon terminals adjacent to

AnkG-labeled axon initial segments are included and provide strong support for the identity of the cells. However, they cannot convey the extensiveness and patterns of the axonal arborizations of these cells.

The intersectional genetic methods included use of the lineage marker *Nkx2.1* with either *Unc5b* or *Pthlh* as the molecular marker. As described, the mice with intersectional targeting of *Nkx2.1* and *Unc5b* appear to show the most specific brain-wide labeling for AACs, and the majority of the descriptions are from these mice. The targeting with *Nkx2.1* and *Pthlh* is less convincing and there appears to be a disconnect between the descriptions and the images. While the descriptions emphasize that the labeling is very similar in the two types of mice, the images suggest distinct differences, including labeling of non AACs in striatum and layer 4 of the cortex in the *Pthlh*;*Nkx2.1* mouse, as described in the manuscript. In addition, the *Pthlh*;*Nkx2.1* mouse has higher cell targeting in some regions and fewer labeled cells in others. Perhaps it would be more accurate to present the *Pthlh*;*Nkx2.1* mouse as differing from the *Unc5b*;*Nkx2.1* mouse, but useful for AAC labeling in select regions and under some conditions, such as following tamoxifen administration at specific ages. As currently presented, the inclusion of the *Pthlh*;*Nkx2.1* detracts from the otherwise convincing argument that the *Unc5b*;*Nkx2.1* mouse provides a specific and comprehensive way to identify AACs.

<https://doi.org/10.7554/eLife.93481.2.sa1>

Reviewer #3 (Public Review):

Summary:

Raudales et al. aimed at providing an insight into the brain-wide distribution and synaptic connectivity of bona fide GABAergic inhibitory interneuron subtypes focusing on the axo-axonic cell (AAC), one of the most distinctive interneuron subtypes, which innervates the axon initial segments of glutamatergic projection neurons. They establish intersectional genetic strategies that enable them to specifically and comprehensively capture AACs based on their lineage (*Nkx2.1*) and marker expression (*Unc5b*, *Pthlh*). They find that AACs are deployed across essentially all the pallium-derived brain structures as well as anterior olfactory nucleus, taenia tecta, and lateral septum. They show that AACs in distinct areas and layers of the neocortex as well as different subregions of the hippocampal formation display unique soma and synaptic density and morphological variations. Rabies virus-based retrograde monosynaptic input tracing reveals that AACs in the neocortex, the hippocampus, and the basolateral amygdala receive synaptic inputs from common as well as specific brain regions and supports the utility of this novel genetic approach. This study elucidates brain-wide neuroanatomical features and morphological variations of AACs with solid techniques and analysis. Their novel AAC-targeting strategies will facilitate the study of their development and function in different brain regions. The conclusions in this paper are well supported by the data. However, there are a few minor comments.

(1) The authors added a description about validation of ChCs in the method section: "Validation was conducted with high-magnification confocal microscopy and defined by a cell exhibiting at least two RFP-labelled axons colocalized with AIS labelled by AnkryinG or Phospho-IkB α ". However, this does not clearly define pAACs themselves. If they follow this criteria, an RFP-labeled cell exhibiting only one synaptic cartridge that is colocalized with an AIS should be a pAAC. Is this what the authors are trying to say?

On the other hand, in the response to reviewers, the authors apparently define pAACs in a different way, in which they more focus on the number of cells exhibiting cartridges that are associated with AISs in a certain anatomical region rather than the number of cartridges per cell.

"For BNST we did not positively identify more than a few exhibiting overlap with AnkryinG/IkBa, so we currently leave them as pAACs"

"Putative AAC (pAACs) refers to populations in which relatively few single cell examples of AACs exhibiting co-localized cartridges were found"

The authors need to directly define pAACs.

(2) In the response to reviewers, the authors claimed that both Pthlh and Unc5b mice are useful for studying developing AACs. It would be nice if they include this content in the text (e.g. Discussion).

<https://doi.org/10.7554/eLife.93481.2.sa0>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public Review):

Summary:

In this manuscript, the authors set out to develop genetic tools that can specifically and comprehensively label Axo-Axonic Cells (AACs), also known as Chandelier cells. These AACs possess unique morphological and connectivity features, making them an ideal subject for studying various aspects of cell types across different experimental methods. To achieve both specificity and comprehensiveness in AAC labeling, the authors employ an intersectional strategy that combines lineage origin and molecular markers. This approach successfully targets AACs across the mouse brain and reveals their widespread distribution in various brain structures beyond the previously known regions. Additionally, the authors utilize rabies transneuronal labeling to provide a comprehensive overview of AACs, their variations, and input sources throughout the brain. This experimental approach offers a powerful model system for investigating the role of AACs in circuit development and function across diverse brain regions.

Strengths:

Genetic Tools and Specificity: The authors' genetic tools show qualitative evidence of specificity for AACs, opening new avenues for targeted research on these cells. The use of intersectional strategies enhances the precision of AAC labeling.

Widespread Distribution: The study significantly broadens our understanding of AAC distribution, revealing their presence in brain regions beyond what was previously documented. This expanded knowledge is a valuable contribution to the field.

Transneuronal Labeling: The inclusion of rabies transneuronal labeling provides a comprehensive view of AACs, their variations, and input sources, allowing for a more holistic understanding of their role in neural circuits.

Weaknesses:

Quantitative Analysis: While the claim of specificity appears qualitatively convincing, the manuscript could be improved with more quantitative analysis.

We are glad that the reviewers appreciated our multimodal and brain-wide characterizations of the AAC population. We include many qualitative AAC examples and would like to highlight the quantitative nature of our whole brain cell body and cartridge analyses, made possible by transgenic targeting and our serial two-photon tomography imaging platform (STP). In addition to providing this brain wide AAC atlas, we also propose AACs as perhaps one of the best case examples for a bona fide cell type, which may inspire further in-depth anatomical and functional studies of AACs, and efforts to capture other ground truth cell types.

Comprehensiveness Claim: The assertion of comprehensiveness, implying labeling "almost all" AACs in all brain regions, is challenging to substantiate conclusively. Acknowledging the limitations of proving complete comprehensiveness and discussing them in the discussion section would be more appropriate than asserting it in the results section.

We thank the reviewer for this suggestion and have revised the results and discussion sections accordingly. The issue of how to access comprehensiveness in AAC labeling is a fair and important point, as dense brain-wide AAC labeling has not been achieved and assessed before. Previous studies had used less efficient and specific methods for capturing AACs, primarily in select areas of cortex, hippocampus, and amygdala. These AAC populations are recapitulated by our genetic strategies with higher density and specificity. It does not seem that we have missed any previously-reported AAC populations; in fact, we discovered multiple previously unreported populations. Another evidence supporting our "comprehensive" labeling of AACs is that two independent *Unc5b* and *Pthlh* transgenic strategies showed very similar AAC distribution patterns (Fig. 1 Suppl. 3). However, we recognize that probably the only way to fully assess "completeness" of labeling may be to compare with anatomical ground truth, such as by dense EM reconstruction of all AACs across the brain volume. This is currently not technically possible but may become feasible in the future.

Local Inputs: While the manuscript focuses on inter-areal inputs to AACs, it would benefit from exploring local inputs as well. Identifying the local neurons that target AACs and analyzing their patterns could provide valuable insights into AAC function within specific brain regions.

This is a good suggestion. However, our serial two-photon tomography imaging platform does not have the capability for reliably preserving tissue sections for immunohistochemical processing afterward. Additionally, though our starter AAV injections were limited to 100-150nL, there were far too many input cells labelled at the injection side to resolve individual input cells and correlate with their synaptic partners (e.g. a rabies-labelled pyramidal cell within the injection site may still project to starter cell few hundred microns away). Thus, our rabies input mapping was best suited for characterizing long-range inputs and was the focus here. For studying local inputs to AACs, future studies could combine very dilute starter AAV injections with multi-marker characterization of cell types by immunohistochemistry or FISH.

Discussion Focus: The discussion section should delve deeper into the biological implications of the findings, moving beyond technical significance. Exploring similarities and differences in input patterns between AACs and other cell types, and linking them to the locations of starter cells or specific connectivity patterns in the brain, would enrich the discussion. For instance, investigating whether input patterns can be predicted based on the locations of starter cells or connectivity specificity could provide valuable insights.

We thank the reviewer for this suggestion. We have expanded the discussion to include more on the relevance and implications of our input mapping results to different starter populations of AACs.

Reviewer #2 (Public Review):

Summary:

The goals of this study were to develop a genetic approach that would specifically and comprehensively target axo-axonic cells (AACs) throughout the brain and then to describe the patterns and characteristics of the targeted AACs in multiple, selected brain regions. The investigators have been successful in providing the most complete description of the regional distribution of putative (pAACs) throughout the brain to date. The supporting evidence is convincing, even though incomplete in some brain regions. The findings should serve as a guide for more detailed studies of AACs within each brain region and lead to new insights into the connectivity and functional organization of this important group of GABAergic interneurons.

Strengths:

The study has numerous strengths. A major strength is the development of a unique intersectional genetic strategy that uses cell lineage (Nkx2.1) and molecular (Unc5b or Pthlh) markers to identify axo-axonic AACs specifically and, apparently, nearly completely throughout the mouse brain. While AACs have been described previously in the cerebral cortex, hippocampus, and amygdala, there has been no specific genetic marker that selectively identifies all AACs in these regions.

The current genetic strategy has labeled pAACs in a large number of additional brain regions, including the claustrum-insular complex, extended amygdala, and several olfactory centers. In general, the findings provide support for the specificity of the methods for targeting AACs, and include some examples of labeling near markers of axon initial segments. However, the Investigators are careful to refer to labeled neurons as "putative AACs" as they have not been fully characterized and their identity verified.

The descriptions and numerous low-magnification images of the brain provide a roadmap for subsequent, detailed studies of AACs in numerous brain regions. The overview and summaries of the findings in the Abstract, Introduction, and Discussion are particularly clear and helpful in placing the extensive regional descriptions of AACs in context.

Weaknesses:

One weakness of the study is the lack of an illustration of the high-resolution cell labeling that can be achieved with the methods, including labeling of numerous rows of axon terminals in contact with axon initial segments. The initial images of the brain-wide distribution of putative AACs are necessarily presented at low magnification. Although the authors indicate that the cells have "highly characteristic AAC labeling patterns throughout the neocortex, hippocampus and BLA", these morphological details cannot be visualized by the reader at the current magnification, even when the images are enlarged on the computer screen. Some of the details become evident in later Figures, but an initial illustration of single cell labeling with confocal microscopy, or tracing of their characteristic axonal arbors, would support the specificity of the labeling in the low magnification images.

We thank the reviewer for the suggestion. We have now added high-resolution images showing the colocalization of AAC axon boutons (cartridges) along AnkG positive

postsynaptic axon initial segments in Fig. 2 Suppl. 1, Figure 1 panels a, d, e, and Fig. 4 panels b, c. These images unequivocally demonstrate AAC identity and specificity.

Table 1 indicates that the AAC identity of the cells has been validated in many brain regions but not in all. The methods used for validation have not been described and should be included for completeness. The authors are careful to acknowledge that labeled cells in some regions have not been validated and refer to such cells as pAACs.

Validation was defined by colocalization of RFP-labelled AAC cartridges and AnkryinG or Phospho-IκBα-labelled axon initial segments, imaged by confocal microscopy. We provide high-magnification examples throughout figures 2-6 and supplements. We have also tried to clarify this better in the methods section entitled “Immunohistochemistry.” Putative AAC (pAACs) refers to populations in which relatively few single cell examples of AACs exhibiting co-localized cartridges were found, largely due to the sparsity of the low tamoxifen dosage used (see response above).

The intersectional genetic methods included the use of the lineage marker Nkx2.1 with either Unc5b or Pthlh as the molecular marker. As described, the mice with intersectional targeting of Nkx2.1 and Unc5b appear to show the most specific brain-wide labeling for AACs, and the majority of the descriptions are from these mice. The targeting with Nkx2.1 and Pthlh is less convincing. The title for Figure 1 Supplemental Figure 3 suggests a similar AAC distribution in the Pthlh;Nkx2.1 mouse compared to the Unc5b;Nkx2.1 mouse. However, the descriptions of the individual panels suggest a number of inconsistencies and non-AAC labeling. The heavy labeling in the caudate and cells in layer 4 is particularly problematic. Based on the data presented, it appears that heavy labeling achieved in these mice could not be relied on for specific labeling of all AACs, although specific labeling could be achieved under some conditions, such as following tamoxifen administration at select ages.

The reviewer is correct about *Pthlh* being less specific for AACs than *Unc5b* when crossed to a constitutive *Nkx2.1* recombinase driver line. *Pthlh/Nkx2.1* intersection labeled a set of layer 4 cells in somatosensory cortex and dense cells in striatum, which are clearly not AACs. But these are the only main difference compared to *Unc5b/Nkx2.1* intersection. As the reviewer points out, it is only when *Pthlh* is crossed to an inducible *Nkx2.1-CreER* line and induced embryonically with tamoxifen that there is more specific AAC labeling (at least in cortex). We included this data as well as the intersection with *VIP-Cre* in case either of these are useful to researchers studying fate-mapping of AACs or bipolar cell interneurons. We have also revised the title of Fig. 1 Suppl. 3 to better convey this.

The methods described for dense labeling and single-cell labeling are described briefly in the methods. Some discussion of the development of the methods would be useful, including how it was determined that methods for heavy labeling identified AACs specifically and completely.

We have added a description on the development of these to the methods section entitled “Animals.”

Reviewer #3 (Public Review):

Summary:

Raudales et al. aimed at providing an insight into the brain-wide distribution and synaptic connectivity of bona fide GABAergic inhibitory interneuron subtypes focusing on the axo-axonic cell (AAC), one of the most distinctive interneuron subtypes, which innervates the axon initial segments of glutamatergic projection neurons. They establish

intersectional genetic strategies that enable them to specifically and comprehensively capture AACs based on their lineage (Nkx2.1) and marker expression (Unc5b, Pthlh). They find that AACs are deployed across essentially all the pallium-derived brain structures as well as the anterior olfactory nucleus, taenia tecta, and lateral septum. They show that AACs in distinct areas and layers of the neocortex as well as different subregions of the hippocampal formation display unique soma and synaptic density and morphological variations. Rabies virus-based retrograde monosynaptic input tracing reveals that AACs in the neocortex, the hippocampus, and the basolateral amygdala receive synaptic inputs from common as well as specific brain regions and supports the utility of this novel genetic approach. This study elucidates brain-wide neuroanatomical features and morphological variations of AACs with solid techniques and analysis. Their novel AAC-targeting strategies will facilitate the study of their development and function in different brain regions. The conclusions in this paper are well supported by the data. However, there are a few comments to strengthen this study.

(1) The definition of putative AAC (pAAC) is unclear and Table 1 may not be accurate. Although the authors find synaptic cartridges of RFP-labeled cells in the claustrinsular complex and the dorsal endopiriform nuclei, they still consider these cells as pAACs (not validated). The authors claim that without examining the presence of synaptic cartridges, RFP-labeled cells in the hypothalamus and the bed nuclei of the stria terminalis (BNST) are pAACs while those in the L4 of the somatosensory cortex in Pthlh;Nkx2.1;Ai65 mice are non-AACs. In Table 1, the BNST is supposed to contain AACs (validated), but in the text, the authors claim that RFP-labeled cells in the BNST are pAACs. Could the authors clarify how AACs, pAACs, and non-AACs are defined?

We thank the reviewer for their interest and comments on our work. Please see our response to reviewer 2 for clarification on putative pAACs. Additionally, we have clarified in the methods under “Immunohistochemistry” how we defined AACs, pAAC, and non-AACs. For BNST we did not positively identify more than a few exhibiting overlap with AnkryinG/IkBa, so we currently leave them as pAACs—Table 1 has been corrected to reflect this.

(2) The intersectional strategies presented in this study could also specifically capture developing AACs. If so, how early are AACs labeled in the brain? It would also be nice if the authors could add a simple schematic like Fig. 1a showing the time course of Pthlh expression.

We thank the reviewer for suggesting the application of our method in studying AAC development. As the onset of *Unc5b* is in early postnatal time, tamoxifen induction of *Unc5b-CreER* in early postnatal days can enable studies of AAC neurite and synapse development, maturation, and plasticity. Similarly, *Pthlh* expression in the brain is relatively low/absent at P4 and present at P14 and later timepoints. *Pthlh-Flp;Nkx2.1-Cre* intersection can be used to study postnatal AAC development and plasticity.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

While the claim of specificity appears qualitatively convincing, additional quantitative analysis would make the authors' claim much stronger. For example in Figure 4 (f-h), where the authors show an overlap of AAC axons with AnkG labeling, there also appears to be a region of AAC axon lacking adjacent AnkG labeling. The author could quantify the fraction of cartridges that overlap with AnkG labeling in different brain regions, potentially stringing their claim that pAACs are AACs as well as providing important documentation of the diversity or homogeneity of compartment targeting across the brain.

As mentioned previously, we only performed AnkG co-labeling analysis on low-dose tamoxifen/sparsely labelled samples in which we could readily differentiate individual cells. This was performed on samples with the Ai65 cytoplasmic reporter—for validation purposes we could positively identify co-labelled cartridges, but it would be more difficult to accurately identify any cartridges not co-labeled (since the entire axon was labelled with RFP). For precisely identifying and mapping AAC cartridge locations we found the intersectional synaptophysin-EGFP reporter (Fig. 2k-n) to be a more precise method for specifically labeling the “cartridge” segment of AAC axons. However, we did not try AnkG staining on samples from this reporter line, as they were set aside for STP imaging.

Regarding the claim of comprehensiveness, labeling “almost all” AACs in all brain regions is a high standard and challenging to demonstrate conclusively. The study already significantly expands our understanding of AAC distribution, and the authors might consider discussing the limitations of proving complete comprehensiveness in the discussion rather than claiming it in the results section.

We again thank the reviewer for this critique. As mentioned above, we have revised the results and discussion sections to better convey this point across.

Furthermore, the manuscript connectivity section primarily focuses on inter-areal inputs to AACs, but it could benefit from exploring local inputs as well. By identifying the local neurons that target AACs, the authors could ask if there is any general property or rule of the local projections to AACs across the brain, or at least within the cortex. Moreover, a clear indication of the injection site would be helpful, particularly in Figure 7, where there seems to be some discrepancy between the histograms and fluorescent images regarding local projections. The histograms of Figure 7, seem to indicate that the local projection to AACs is a small fraction of all the presynaptic neurons, however, the fluorescent image for the SSp seems to suggest otherwise with many fluorescent cells in the injected area.

We thank the reviewer for these comments. Regarding the local inputs in the rabies tracing datasets, it is a limitation (as mentioned above) of our STP platform’s inability to preserve tissue for immunohistochemistry labeling as well as our relatively dense starter cell labeling. Instead, our focus here was on long-range inputs (i.e. outside the ipsilateral ARA area of injection), which was simply not known for these AAC populations. We have revised the Figure 7 legend and added a description in the methods section to more clearly indicate that we only included long-range input projections in the Figure 7 histograms.

In the discussion, the authors should delve more into the biological implications of their findings rather than solely emphasizing the technical significance. They could explore the similarities and differences in input patterns between AACs and other cell types, potentially linking them to the locations of their starter cells or specific connectivity patterns in the brain. For example, the authors could check if the input patterns could be predicted from the projections to the layers where their starter cells are located (either from an Atlas like the Allen Connectivity Atlas, or from retrograde rabies injections in the same locations). Can the differences between the input patterns to PVC and AAC be predicted for their location versus some specificity of connections?

Thank you for the extensive comment. We address this point above, and have revised our discussion accordingly.

Reviewer #2 (Recommendations For The Authors):

The Figure legends vary in completeness and quality.

(1) The legend for Figure 1 is very informative, and section e-g serves as a useful guide, as the legend includes the names of the brain regions related to the abbreviations and also indicates the specific panels that show the identified structures. Because of the large number of structures and the number of panels in each Figure, it would be ideal to follow the same pattern in the remaining figures.

(2) Several edits are needed in the legend for Figure 1 Supplement Figure 1. The descriptions of a-f could be improved by providing general terms to describe the brain regions associated with the latter list of abbreviations (as has been done with the identification of the cerebral cortex, hippocampus, and olfactory centers and their related panels). One suggestion would be to write out insula, claustrum, and endopiriform prior to listing the abbreviations (AI, CLA, EP) (b-c) and adding amygdaloid complex and extended amygdala before the abbreviations (COA, BLA, MeA) (d-f) and (BST) (d).

We thank the reviewer, as the suggestion of further expanding the abbreviations is a good one. As such, we have revised/reorganized the anatomical abbreviations in the figure legends for Figure 1 Supplement Figures 1, 2, and 3.

Descriptions for Panels g-j require editing to link the appropriate panels and the descriptions. Panels for BSTpr appear to be g-h (rather than f-g) and i,j (rather than h-i).

We have fixed this typo in the legend for Figure 1 Supplement Figure 1.

Descriptions for Panels k-n could be edited to include abbreviations for the identified brain regions. For example, include the abbreviation ARHP after arcuate nuclei and indicate panels m-n (rather than j-l); include PVP after paraventricular and indicate panel n (rather than m); include DMPH after dorsomedial nuclei and indicate k-m (rather than j-l).

Thank you for the suggestion. We have expanded the abbreviations in Figure 1 Supplement 1 accordingly.

Reviewer #3 (Recommendations For The Authors):

(1) Please clarify if tdTomato, EGFP (from helper AAVs), and RFP (from rabies virus) are native signals or IHC signals in legends.

We have added the descriptors “native” or “stained” to all figure legends containing fluorescent images.

(2) Fig. 4b and c: Please add insets of high-magnification images showing AAC boutons along AnkG-labeled AISs.

We have added these insets to Fig. 4b and c.

(3) Fig. 7S1: It appears that d and e are reversed. Judging from the positions of starter cells, d is for PV-Cre? Please make sure. It is also better to draw the laminar border in d and e.

The original genotype labels are correct for Fig. 7S1 d and e. We have added the laminar borders as suggested.

(4) Fig. 9b: Just for consistency, please label with the name of the helper AAV.

Added.

| (5) Line 617: *intragranular>>>infragranular?*

Corrected, thank you.

| (6) *It may be unclear to some readers if the images in the figures are from confocal or STP. The authors may want to clarify that all images in the figures are generated by confocal microscopy in the method section.*

We have clarified this better in the methods section, “Microcopy and image analysis.”

| (7) *The authors should clarify that STP was used to map input cells to the brain in the result section.*

We have added this description in the results section.

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