Id2 GABAergic interneurons comprise a neglected fourth major group of cortical inhibitory cells

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Summary

Cortical GABAergic interneurons (INs) represent a diverse population of mainly locally projecting cells that provide specialized forms of inhibition to pyramidal neurons and other INs. Most recent work on INs has focused on subtypes distinguished by expression of Parvalbumin (PV), Somatostatin (SST), or Vasoactive Intestinal Peptide (VIP). However, a fourth group that includes neurogliaform cells (NGFCs) has been less well characterized due to a lack of genetic tools. Here, we show that these INs can be accessed experimentally using intersectional genetics with the gene \textit{Id2}. We find that outside of layer 1 (L1), the majority of \textit{Id2} INs are NGFCs that express high levels of neuropeptide Y (NPY) and exhibit a late-spiking firing pattern, with extensive local connectivity. While much sparser, non-NGFC \textit{Id2} INs had more variable properties, with most cells corresponding to a diverse group of INs that strongly expresses the neuropeptide CCK. \textit{In vivo}, using silicon probe recordings, we observed several distinguishing aspects of NGFC activity, including a strong rebound in activity immediately following the cortical down state during NREM sleep. Our study provides insights into IN diversity and NGFC distribution and properties, and outlines an intersectional genetics approach for further study of this underappreciated group of INs.
Introduction

The neocortex is comprised of two primary neuronal classes: pyramidal cells that release the excitatory neurotransmitter glutamate, and interneurons that release the inhibitory neurotransmitter GABA. Pyramidal neurons project their axons locally and distally to form extended circuit ensembles across cortical and subcortical areas, whereas GABAergic interneurons (INs) typically project their axons locally to regulate the excitability of nearby pyramidal cells and other INs. Pyramidal neurons share a common dendritic structure characterized by an apical dendrite that typically extends up towards the pial surface with extensive arborization in cortical layer 1 (L1), a cell sparse region that receives diverse long-range cortical and thalamic axonal projections (1-4). In combination with its basal dendrite and somatic inputs, the pyramidal neuron thus has the ability to distinguish and integrate inputs from bottom-up and top-down sources in real-time, a key feature that enables dynamic cortical activity and adaptability during the animal’s interactions with the external world (4, 5).

While the morphological diversity of IN dendrites and axons was noted over a century ago by Ramón y Cajal, recent work has begun to elucidate how this specialization of IN subtypes allows for compartment-specific inhibition across the entire pyramidal neuron (6-11). The discovery of molecular markers in tandem with electrophysical and morphological approaches has enabled the delineation of several major IN groups and subtypes, each with distinctive roles in the cortical circuit (6, 12-18). INs that express the Ca^{2+} binding protein parvalbumin (PV), which account for ~40% of the GABAergic INs in neocortex, exhibit fast-spiking properties, with most projecting their axons to form perisomatic baskets on nearby pyramidal neuron soma.
In layer 4 of sensory cortical areas, these PV INs form a canonical circuit in which they provide feedforward inhibition of excitatory neurons receiving core thalamic inputs (20, 21). A distinct but less abundant subtype of PV INs are the chandelier cells, whose axons form cartridge-like inputs on the pyramidal neuron axon initial segment, thereby enabling control and coordination of the axonal output of pyramidal neuron ensembles (22-24). Somatostatin (SST) expressing interneurons (accounting for ~30% of the INs) represent a second major group of INs, with the majority characterized by an ascending axonal arbor that targets the distal apical dendrites of pyramidal cells (SST Martinotti cells) (6, 25). A third major group of INs is distinguished by the expression of the neuropeptide vasoactive intestinal peptide (VIP); these interneurons account for ~12% of the INs in the neocortex and include cells that preferentially target other interneurons, particularly SST INs, and thus act to disinhibit the local circuit (26-29). The vast majority of recent studies exploring the roles of INs in cortical function have focused on these three groups, in no small part due to the establishment of genetic tools in rodents for targeting and manipulating the activity of each group (12, 30-32). This body of work has revealed how IN diversity facilitates greater flexibility and computational power in cortical circuits.

In addition to the three main IN groups described above, there remains an additional population of GABAergic interneurons (about 18% of the INs in the neocortex) that have been much less studied. This IN population is particularly enriched in superficial layers of the neocortex, and includes ~90% of the INs in layer 1 (6, 33) as well as neurogliaform cells (NGFCs) in all layers and CCK basket cells (CCK BCs), two IN types previously identified in the neocortex that do not express PV, SST or VIP (34-
NGFCs are an interneuron type that exhibits several remarkable properties, including an extremely high degree of output connectivity to other cells (pyramidal neurons and other INs) and the ability to induce postsynaptic GABA-B responses following a single action potential (38-41). CCK BCs are a rare species in the neocortex whose synapses on pyramidal neurons exhibit endocannabinoid mediated depolarization induced suppression of inhibition (DSI) (37, 42).

Cortical GABAergic INs are specified during embryogenesis in the medial and caudal ganglionic eminences of the ventral telencephalon (MGE and CGE, respectively) (43-45), with a fraction of non-MGE derived INs originating from the preoptic area (POA) (46). All cortical PV and SST INs arise from Nkx2.1+ progenitors within the MGE (47), and express Lhx6 during tangential migration and in the adult (48). Cortical CGE derived INs, i.e., the non-PV, non-SST subtypes labeled in the Htr3a (BAC)-EGFP mouse line (the 5HT3aR INs) (49) can be divided into two primary populations: VIP- and non-VIP-expressing (6, 50, 51). While transgenic targeting approaches for PV, SST, and VIP INs have been developed and widely implemented (12, 31), accessing non-VIP CGE (or 5HT3aR) INs – a fourth major IN group – has been challenging due to a lack of genetic strategies to target this population, and therefore the IN subtypes present in this population are not well characterized.

Here, we show that Id2 is a marker for the INs in the neocortex that do not express PV, SST or VIP, including the non-VIP cells in L1, NGFCs in L2-6 and a variety of CCK INs. Using intersectional genetic strategies to label and optogenetically stimulate Id2 INs, we find that the vast majority of Id2 INs in L2-6 are NPY-expressing NGFCs that exhibit the late-spiking firing pattern and broad connectivity characteristic of
these cells. In contrast, the much rarer non-NGFC Id2 INs display diverse firing patterns and DSI but sparse local connectivity. Combining opto-tagging with silicon probe recordings, we observe several intriguing features of NGFC in vivo behavior, such as a robust rebound in activity immediately following the cortical down states during NREM sleep. Thus, our work furthers the study of IN diversity and enables genetic access to a group of INs that despite their potent GABAergic output have remained somewhat overlooked to date.
Results

We examined cortical single-cell transcriptome data (scRNAseq) from the Allen Institute (14) and observed that expression of the Id2 gene was highly enriched in the INs in their collection that do not express PV, SST or VIP (Figure 1A) (52). The Id2 IN population includes cells that express markers known to be present in non-VIP CGE INs such as Reelin (53), and NPY (54), as well as the more recently identified markers NDNF, Lamp5 and Sncg (14). Updated and expanded cortical and hippocampal scRNAseq data from the Allen Institute (18, 55) confirms that Id2 is a useful marker for studying non-PV, SST, or VIP INs (Figure 1 – figure supplement 1). To develop a transgenic approach to target Id2 INs, we took advantage of an existing Id2-CreER driver line (56) that expresses a tamoxifen-inducible form of Cre recombinase (57). Since Id2 is also expressed in pyramidal and non-neuronal cell types in the cortex, we crossed the Id2-CreER driver with a pan-IN Flp driver (Dlx5/6-Flpe) (53) such that only Id2 INs would be targeted with intersectional reporters (Figure 1B). In combination with the Cre and Flp dependent tdTomato reporter Ai65 (30), this intersectional genetics approach yielded labeling of INs in all cortical layers of the somatosensory cortex barrel field (S1BF) following tamoxifen administration (3-4 doses between P21-P35; see Methods) to activate the CreER (Figure 1C).

Histological analysis of Id2 INs. To determine the proportion of Id2 vs. non-Id2 INs, we utilized the intersectional/subtractive reporter FLTG (58) where Flp activity results in tdTomato expression, but Flp + Cre results in EGFP labeling (Figure 1D). Quantification of Id2 INs (green) and non-Id2 INs (red) in the S1BF of well-labeled brains (see Methods) yielded an overall fraction of 18% (680/3812 cells, n=4 brains), with the
majority of Id2 INs located in superficial layers (L1-3; Figure 1E-F). This proportion and
distribution was consistent with our previous estimates for non-VIP 5HT3aR or CGE INs
(6), indicating that this genetic strategy was efficiently targeting this population. We
observed a similar overall proportion of Id2 INs in other sensory areas (e.g., V1),
consistent with a recent comprehensive survey of interneuron subtype distribution (18),
as well as in a higher order cortical area, the prelimbic cortex (18%; Figure 1 – figure
supplement 2).

To assess any potential overlap between Id2 INs and PV, SST, or VIP INs, we
performed intersectional genetic labeling experiments with the respective Flp drivers
that cover those populations. Both PV and SST INs originate from Nkx2.1+ progenitors
(i.e., MGE lineages; (47)), and are efficiently labeled using an Nkx2.1-Flpo driver line
(31). However, there is also a population of neurogliaform INs (NGFCs) that arise from
an Nkx2.1+ lineage, the vast majority of which migrate to the hippocampus (59). A small
fraction of these MGE-derived NGFCs is located in the neocortex; these are the sparse
deep layer Lamp5/Lhx6 cells described and characterized previously by us using
intersectional genetics with Id2 (Id2-CreER; Nkx2.1-Flpo; Ai65) (60, 61). Id2/Nkx2.1
cells did not show any overlap with PV or SST, and were mostly localized in L6 (61)
(Figure 1 – figure supplement 3). To examine possible overlap with VIP populations, we
generated Id2-CreER; VIP-Flpo; Ai65 animals and assessed the degree of IN labeling.
Here too, we observed very few labeled cells in S1BF, with most being located along
the L1/2 border (Figure 1 – figure supplement 3). While Id2/VIP cells were extremely
sparse in S1BF, they were somewhat more prevalent in the prelimbic cortex along the
L1/2 border (Figure 1 – figure supplement 3). Thus, we find that the vast majority of Id2
INs (>95%) are distinct from PV, SST or VIP populations, and in the cortex, almost all originate from non-Nkx2.1 lineages (i.e., from the CGE/POA).

In superficial layers (L1-3), expression of neuropeptide Y (NPY) has been utilized successfully as a proxy to identify NGFCs (33, 54, 62, 63). We evaluated the overlap between Id2 INs and NPY by performing fluorescence ISH (FISH) for tdTomato and NPY on tissue cryosections from Id2-CreER; Dlx5/6; Ai65 brains (S1BF; Figure 2A-B). The proportion of Id2/NPY cells vs. the total Id2 labeled population in S1BF ranged from 29% in L1 to 82% in L2/3, 95% in L4-5, and 61% in L6, with a combined proportion in L2-5 of 87% (638 cells counted, sections from three brains), suggesting that in contrast to L1, where Id2 INs consist of several subtypes (33), the vast majority of Id2 cells outside of L1 are NGFCs. Within L1, our findings are consistent with our previous analysis that about 30% of L1 INs are NPY-expressing NGFCs, with the remainder of the Id2 population being comprised of canopy cells (NDNF/non-NPY) and α7 cells (33).

We further confirmed that most Id2 INs in L2-6 are NPY-expressing throughout different sensory cortical areas by generating Id2-CreER; Dlx5/6-Flpe; Ai65; NPY-hrGFP animals and comparing labeling in S1BF, V1 and A1 (Figure 2 – figure supplement 1).

To explore the nature of the non-NPY Id2 neurons in L2-6, we examined the overlap between Id2 labeled cells and relatively high levels of CCK expression as the latter is a proxy for a sparse population of INs that include the large CCK basket cells (64-66). Using IHC for CCK (Figure 2C-D), overall, in L2-6 of S1BF we observed that 15% of Id2 labeled INs exhibited strong CCK expression (165/1123 cells, sections from three brains), with Id2/CCK cells being most abundant in L2 (14% of Id2 cells) and L6 (25%). No overlap was observed between strong CCK IHC and NPY-hrGFP expression,
consistent with previous work (67) and the current Allen scRNAseq data (Figure 1 – figure supplement 1)(18). Thus, a significant fraction of the Id2/non-NPY population in cortical layers outside of L1 appear to be CCK+ IN subtypes (~80% in L2/3), such that Id2/NPY and Id2/CCK populations account for nearly all of the Id2 INs in L2-5.

Electrophysiological and morphological properties of Id2 INs. Our FISH analysis (Figure 2A-B) suggested that the majority (87%) of Id2 INs in L2-5 are NPY-expressing NGFCs. To test this hypothesis, we characterized the firing properties of Id2 INs in L2-5 of S1BF in acute brain slices. The cells were filled with biocytin during electrophysiological recording for post-hoc morphological analysis. NGFCs in L2/3 and L1 have been shown to have a late-spiking (LS) firing pattern characterized by delayed firing following a slow ramp depolarization when depolarized with step current injections to near-threshold membrane potentials (6, 33, 38, 40, 41). NGFCs in these layers were also found to fire spike trains with little spike frequency adaptation, or even spike frequency acceleration, during depolarizations close to threshold, and mild adaptation during suprathreshold depolarizations. Action potentials in NGFCs have a large AHP and a slow ADP (33, 38, 40, 68-70). We found that in a sample of randomly recorded Id2 cells (n=76), the large majority in L2-5 had a LS firing pattern (36/45; 80% in L2/3 and 28/31; 90% in L4-L5) (Figure 3). These proportions are similar to the proportion of Id2/NPY cells (Figure 2). The Id2 LS cells also showed weak spike frequency adaptation during near threshold membrane depolarizations and had other electrophysiological properties typical of NGFCs (Table 1). In paired recordings of L2/3 LS Id2 cells and PCs, we observed a very high connection probability (70%; 19/27 pairs tested), consistent with previous observations and the hypothesis that NGFCs mediate
volume transmission of GABA (40). Finally, the decay of the IPSC elicited by NGFCs is known to be prolonged compared to that mediated by other INs (38, 39). Consistent with this, we found that the decay rate (80 to 20% of peak) of the IPSC in LS-PC pairs was significantly slower than that in non-LS-PC pairs (LS-PC: 51±8 ms, n=6 pairs; non-LS-PC: 16±3 ms, n=5 pairs; p=0.005, two-tailed unpaired t-test).

Morphologically, the LS cells in L2-5 had the characteristic features of NGFCs with short multipolar dendrites emanating around the cell body and a significantly larger, dense axonal arbor surrounding the cell body and dendritic arbor (Figure 3A-B and Figure 3 – figure supplement 1; n=15), consistent with previous descriptions of NGFC in these layers across several neocortical areas (38-41, 70). NGFCs in L1 resemble NGFCs in L2-5 in having short dendrites and dense axonal arborization, but their axonal arbor extends for longer horizontal distances, spanning several columns (10, 11, 33, 68, 71, 72) and have been called “elongated neurogliaform cells” (10, 11). We found that in the LS cells located in deeper layers, the axonal arbor was also asymmetrical, but often extended in the vertical (columnar) direction. These differences between NGFCs across cortical layers are also evident in the NGFC morphologies illustrated in a recent patch-seq study (17).

The remaining Id2 cells (~20% in L2/3 and ~10% in L4-5) had either an irregular or a bursting firing pattern during low to moderate depolarizations (Figure 3C-D, 3F, and Table 1) and narrower action potentials (Figure 3E). We also observed that a small minority of the non-LS Id2 cells in L2/3 (~15%) were likely α7 cells, a L1 Id2 IN subtype typically localized in the deeper part of L1, which has a bursting firing pattern that can be distinguished from other L2/3 bursting cells by the properties of the burst,
significantly lower input resistance, and morphology (Figure 3 – figure supplement 1) (33). The histological analysis showed that the majority (~80% in L2/3) of the non-NPY Id2 cells strongly express the neuropeptide CCK (Figure 2). CCK is expressed at varying levels by many types of cortical neurons, including GABAergic and glutamatergic cells; however, it is particularly high in a group of GABAergic interneurons known as CCK-CB1R interneurons or CCK basket cells that have been described in the hippocampus and L2/3 of the neocortex (36, 42, 64, 73, 74). Although much less abundant than other GABAergic interneurons in the neocortex, they are of special interest because of their unique synaptic properties (73, 75, 76). In particular, these cells express CB1 cannabinoid receptors in their presynaptic terminals that mediate a phenomenon known as depolarization-induced suppression of inhibition (DSI) (77, 78). Depolarization of a connected pyramidal cell leads to the release of endocannabinoids from the pyramidal cell that then bind to CB1 receptors located on the presynaptic terminals of the CCK basket cells, leading to suppression of GABA release (79-81).

To test the hypothesis that non-LS Id2 neurons include CCK basket cells, we first examined the effect of depolarization of a pyramidal cell on the PSP generated on L2/3 PCs by light stimulation of Id2 neurons expressing the engineered channelrhodopsin variant CatCh (Id2-CreER; Dlx5/6-Flpe; Ai80; Figure 4A-C)(32, 82). Depolarization of the PC reduced the synaptic response by ~20%, and this reduction was prevented by application of the CB1 receptor antagonist AM251 (Figure 4B-C) suggesting that some of the presynaptic cells innervating the cell had undergone DSI. In paired recordings of L2/3 LS Id2 cells and PCs, we found that the synaptic response elicited by NGFCs did not undergo DSI (n=9; Figure 4E). In contrast, in paired recordings of non-LS Id2 cells
(of 46 nonLS-PC pairs tested, only 9 (20%) were connected), we observed DSI in 3 out of the 5 pairs tested (Figure 4E). Of the cells showing DSI, two were irregular spiking and one was bursting; of the two cells that did not show DSI, one was burst spiking and the other was a putative α7 cell. These results indicate that the partial suppression of GABA release observed when the whole population of Id2 cells is stimulated is occurring primarily on the terminals of the non-LS Id2 cells. Consistent with this, the proportion of the GABAergic response elicited by light activation of Id2 cells that was suppressed by depolarization of the post-synaptic PC is similar to the representation of non-LS cells in the L2/3 Id2 population. These observations, together with the molecular analysis, suggest that the majority of the non-LS Id2 cells are CCK basket cells. The morphology of IS and BS non-LS Id2 cells was similar, but quite distinct from the NGFCs, with significantly longer dendrites and a less symmetrical and much less dense axonal arborization (Figure 3C-D and Figure 3 – figure supplement 1), consistent with the published morphologies of CCK basket cells in the neocortex (36, 74).

**In vivo properties of Id2 INs**

Although NGFCs are a substantial population of GABAergic INs, particularly in the superficial layers of the cortex (where they are slightly more abundant than SST INs), the lack of genetic strategies to study these cells has limited the characterization of their in vivo properties. Nevertheless, given their broad output connectivity, these neurons are likely to contribute significantly to inhibition throughout the cortical column. We utilized our intersectional genetics approach to express CatCh in Id2 INs (Id2-CreER; Dlx5/6-Flpe; Ai80; also see Figure 4) in order to optogenetically identify and stimulate these cells in multichannel silicon probe recordings in the posterior parietal
cortex and V1 of freely moving and behaving animals (Figure 5A). Single units were isolated (n = 571 units from 5 mice) and separated into putative pyramidal cells (PC), narrow waveform (NW) INs and wide waveform (WW) INs (83). Id2 INs expressing CatCh were distinguished from other cells adjacent to the probes by measuring their firing rates in response to light stimulation (Figure 5B). Applying stringent filtering criteria (see Methods), we selected 11 Id2 INs that exhibited robustly increased firing following light stimulation for further analysis (Figure 5B-C). We then characterized the firing properties of these Id2 INs identified in vivo in relation to PCs and other INs. These Id2 INs exhibited WW characteristics, and were readily distinguishable from NW cells, previously identified as PV fast-spiking INs and some SST INs (61) (Figure 5B and D). Consistent with this, these 11 Id2 INs had wider auto-correlograms than other PC and NW INs, similar to the overall WW group (Figure 5E). These results indicate that the previously described WW group (83) includes an unknown proportion of Id2 INs.

In addition to the 11 WW Id2 INs described above, we also observed two light responsive Id2 INs with narrower waveform properties, although these cells did not pass the selection criteria for further analysis. We estimate that the majority of Id2 INs that could be identified by optotagging in our silicon probe experiments were located in deeper layers (~L3-5), where the proportion of Id2 cells that are NGFC is >90% (Figure 2). Thus, we tentatively conclude that the 11 Id2 WW INs we selected for analysis are NGFCs, and that the Id2 cells exhibiting narrower waveform properties are likely to be non-NGFC CCK+ types.

The firing rates of PC, NW and WW neurons varied across behavioral states, typically showing lower activity during sleep (Figure 5F). In contrast, putative Id2 NGFC
INs exhibited relatively consistent firing rates across all conditions, with moderately increased activity during REM sleep (Figure 5F). During NREM sleep, the cortex interleaves periods of high spiking activity (up-states) with transient silences (down-states) (61, 84). To gain insights into the nature of the firing dynamics of Id2 INs during sleep, we aligned the activity of all recorded neurons to the peak of the down-state (Figure 5G). Most of the neurons decreased their activity during down states, and steadily recovered their baseline activity after the down-state (post-down; Figure 5H).

Putative Id2 NGFCs decreased their activity during down states to a similar extent as the other cell groups, but strongly increased their firing above baseline levels immediately following the termination of the down-state (Figure 5H, bottom; ~4 s.d. above pre-down rate for Id2; 0.2, 0.6 and -0.03 for PC, NW and WW, respectively). This rebound in activity was observed in the majority of the putative Id2 NGFCs (Figure 5G, right; 64% of the Id2 NGFCs vs. 7%, 14% and 1% for the PC, NW, and WW interneurons, respectively; p < 10^{-10}, chi-squared test) and was negatively correlated with the degree of firing suppression during down-states for the Id2 group (Figure 5I), suggesting that intrinsic membrane properties of the Id2 INs could be mediating, at least in part, their increased activity after down-states. No other neuron group showed a significant correlation between down and post-down state activity, notwithstanding the considerably bigger sample size (Figure 5I). Furthermore, Id2 INs fired at an early position during the up-states sequences (Figure 5J), as estimated by the normalized rank order during the detected up-state epochs (see Methods).

Finally, to understand how Id2 IN activity impacts cortical circuit dynamics *in vivo*, we performed optogenetic stimulation of the Id2 IN population and analyzed which
fraction of putative PC, NW, or WW were suppressed (neg mod in Figure 6A), activated (pos mod) or unchanged (no mod). Light stimulation resulted in a significant decrease in firing rate for about half of the population of all considered cell types (Figure 6A), consistent with the broad direct inhibition expected from NGFCs. PCs in the supra-granular layers were more frequently suppressed than deeper layer PCs, while both NW and WW INs showed the opposite trend across lamina. Interestingly, we also observed a slow positive activity modulation of a subset of INs, and to a lesser extent PCs, following stimulation of Id2 INs (Figure 6A; darker quantiles in Figure 6B-C). Positively modulated responses consistently lagged the negative responses (Figure 6B-D), suggesting an indirect disinhibitory mechanism for the positive responses. Overall, we find that the recruitment of Id2 INs leads to a wide range of response dynamics throughout the cortical column, including both inhibitory and to a lesser extent, relatively slower disinhibitory effects.
Discussion

The use of molecular markers to classify subpopulations of cortical GABAergic interneurons (INs) has provided deep insights into IN diversity (6, 66, 85, 86), and has expanded in recent years with the advent of single-cell RNA sequencing methods to profile IN transcriptomes (14, 16, 18, 87). Identification of differentially expressed genes has enhanced our ability to design and implement genetic strategies to label and experimentally manipulate defined populations of INs in order to understand their specialized functions in cortical circuitry. However, several critical issues have emerged from this body of work that require consideration. Differentially expressed genes that exhibit distinct labeling patterns as assessed by immunohistochemistry (IHC) or in situ hybridization (ISH) may also be expressed at lower levels in broader cell populations that escape detection with those methods. In transgenic animals expressing recombinases such as Cre, labeling strategies that utilize viral or genetically encoded Cre-dependent reporters typically reveal these additional populations due to the lower expression threshold required for labeling with these genetic methods compared to IHC or ISH. For example, expression of the marker CCK as assessed by IHC or ISH has been used to identify subsets of INs with relatively high levels of CCK, but the use of CCK-Cre with a genetic reporter results in much more extensive labeling of INs (Figure 1 – figure supplement 4) as well as pyramidal cells.

Another important consideration is the developmental trajectory of marker gene expression, in that the adult expression pattern for a particular marker may be much more restricted than its cumulative expression. This appears to be the case for the marker 5HT3aR (Htr3a), where expression of EGFP from a BAC transgenic mouse line
labels virtually all non-PV or SST INs (i.e., all of the CGE-derived INs), a finding that led to the conclusion that PV, SST and 5HT3aR INs account for all the INs in the neocortex (49, 50). We compared labeling efficiencies obtained with a knock in Htr3a-Flpo line (33) with that of an Htr3a(BAC)-Cre line (88) and the Htr3a(BAC)-EGFP line used by Lee et al. (49), and found that two copies of the Htr3a-Flpo driver could largely recapitulate the cell labeling observed with the higher copy number BAC transgenic lines (Figure 1 – figure supplement 5). This result, along with ISH images of Htr3a expression at embryonic and adult ages (e.g., Allen Brain Atlas) is consistent with broad but transient Htr3a expression in non-PV/non-SST INs that then becomes restricted to a subpopulation of these INs (89).

Use of BAC transgenics has turned out to be more complicated than expected since each transgenic founder harbors its own positional and copy number variation. The Htr3a(BAC)-EGFP founder line was specifically chosen over others for high levels of EGFP expression. In hindsight, this likely contributed to its pan-CGE IN expression pattern, whereas endogenous Htr3a expression appears to be broad but transient in most CGE INs following their specification, being largely restricted to VIP/CCK and Sncg IN subtypes at adult ages. In contrast to the Htr3a(BAC)-EGFP line, the Htr3a(BAC)-Cre founder was selected based on its cumulative recombination pattern with the cre-dependent tdTomato reporter Ai9, and likely has a much lower copy number or genomic insertion location that results in its expression being closer to the endogenous Htr3a gene. The Htr3a-Flpo line is a single copy knock in whose cumulative labeling with the tdTomato reporter Ai65F is less efficient overall than that
seen with either BAC transgenic line, most likely due to the lower/transient expression of Flpo in most CGE INs.

Leveraging the scRNAseq resources provided by the Allen Institute (14), we identified Id2 as a marker for the vast majority of the INs that do not express PV, SST, or VIP. While Id2 is also expressed in pyramidal neurons and several non-neuronal cell types, we employed intersectional genetics with a pan-IN driver (Dlx5/6-Flpe) to restrict labeling to Id2 INs. This labeling approach relies on the use of an Id2-CreER driver, which has its own pros and cons: tamoxifen induction of cre activity in Id2 cells in P21 or older animals avoids spurious cell labeling arising from developmental Id2 expression, but the efficiency of CreER-mediated recombination can be highly variable from animal to animal depending on the effectiveness of tamoxifen administration. Overall, in well-labeled brains, we found that Id2 INs comprised ~18% of the total IN population in S1BF, which is consistent with our previous estimates for the prevalence of non-PV/non-SST/non-VIP INs (6). In L1, a layer consisting only of GABAergic INs, we previously identified three distinct subpopulations of non-PV/non-SST/non-VIP INs: NGFCs, canopy cells, and α7 cells, which together accounted for ~90% of the neurons in this layer, with the remaining 10% consisting of VIP INs (33). Using our Id2 intersectional genetic strategy, we find that 83% of L1 INs are labeled (Figure 1E), close to the expected 90% of non-PV/non-SST/non-VIP INs, suggesting that all non-VIP INs in L1 are Id2 cells.

In L2-5, we find that Id2 INs are mostly comprised of NGFCs (~87%), with the remainder consisting of a diverse CCK+ IN population. While to date NGFCs have mainly been recorded in superficial layers (L1-3), here we observe that NGFCs are
present in all layers, including deep layers, but are enriched in L2/3. On the other hand, CCK+ INs are mainly located in superficial L2/3 along the border with L1 and in L6. As previously shown for the 5HT3aR INs (6), compared to L4-5, the proportion of Id2 INs increases in L6, where this group accounts for about 15% of all INs (Figure 1). Within the L6 Id2 population, about 60% were NPY+ (putative NGFCs), and about 25% were strongly CCK+, with about 10% unaccounted for (Figure 2). Within the Id2 NGFC in L6, a subset originates from the MGE (i.e., Nkx2.1 lineage) and exhibit a striking anti-correlated activity pattern, with peak activity during cortical down-states (61). We did not observe down-state active cells in our Id2 IN recordings (Figure 5G), indicating that NGFCs of CGE origin (the majority of cortical NGFCs) behave in a distinct manner in vivo. Thus, like L1, L6 is a specialized case that merits its own study to elucidate the diverse IN species that reside there.

The massive scale of a recent transcriptomic cell type survey from the Allen Institute (18) offers an unprecedented look at neocortical and hippocampal IN diversity, and presents the opportunity to assess the expression profile of specific genes across all IN types. Focusing on the “CGE” branch of interneurons, we find that Id2 expression largely encompasses the “Lamp5” and “Sncg” branches of their online dendrogram (Figure 1 – figure supplement 1). However, it is important to note that neither Lamp5 or Sncg are uniformly expressed across the different subtypes in their respective branches. In the case of Sncg, many of the CCK+ subtypes within that branch express low if any Sncg mRNA itself (e.g., within the Serpinf1 sub-branch). Furthermore, there is substantial overlap with VIP in the Sncg population overall (~50% of cells), but this VIP/Sncg population does not appear to be well labeled using our Id2 strategy (Figure 1
– figure supplement 3), likely due to the lower levels of Id2 in these cells (Figure 1 – figure supplement 1). The recent development of new drivers for Lamp5 (Lamp5-Flpo; Jax #037340) and SnCg (SnCg-Flpo; Jax#034424) (90) will enable new transgenic targeting approaches for these respective IN populations, but the breadth and specificity of labeling achieved with these new driver lines remain to be determined. Future work will hopefully resolve how this relatively sparse but remarkably diverse CCK+ IN population functions within the cortex, and whether some of these cells might relate to primate-specific CCK+ IN types such as the rosehip cell (91).

The genetic strategies described here have also facilitated the in vivo recording of NGFCs. Outside of L1, where NGFCs express NDNF, a gene that has been used to facilitate the identification and in vivo recording of these neurons (92-95), recording of NGFCs has been limited to blind recordings followed by post-hoc identification (96-98), a very low yield approach. Here, we combined optogenetic labeling via Id2 intersectional genetics with silicon probe recordings to identify putative NGFCs in vivo (Figure 5). We identified 11 Id2 INs located approximately in L3-5 where the vast majority (>90%) of Id2 INs are NGFCs, as defined by histology and electrophysiology (Figures 2-4). We found that these putative NGFCs have a number of unique properties, including relatively constant firing rates throughout different brain states and a strong rebound in activity immediately following the down-state during NREM sleep. We also found that optogenetic stimulation of Id2 INs produced broad inhibition of PCs and other INs, with roughly half of all cells across cortical layers exhibiting a decrease in activity. The notable rebound in NGFC activity following the down-state, together with the extensive output connectivity of these neurons, suggests an important role for this form
of GABAergic inhibition in setting the stage for new incoming information during bouts of
cortical activity. Future work will certainly continue to elucidate the function of this
distinctive GABAergic cell type in regulating cortical circuitry.
## Key Resources Table

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**Mice**

The following primary lines of mice were used to generate the compound transgenic crosses described in this paper: Id2-CreER (Jax #016222) (56), Dlx5/6-Flpe (Jax #010815) (53), NPY-hrGFP (Jax #006417) (99), Nkx2.1-Flpo (Jax #028577) (31), VIP-Flpo (Jax #028578) (31), Ai65 (Jax #021875) (30), Ai80 (Jax #025109) (32), and FLTG (Jax #026932) (58). The reporter lines Ai65, Ai80 and FLTG were maintained as homozygous stocks prior to crossing with compound heterozygous driver lines (e.g., Id2-CreER; Dlx5/6-Flpe X Ai65 homozygous), with the resulting pups genotyped for Cre and Flpe to obtain experimental animals (e.g., Id2-CreER; Dlx5/6-Flpe; Ai65). To generate Id2-CreER; Dlx5/6-Flpe; Ai65; NPY-hrGFP animals, a stock of Ai65 (homozygous); NPY-hrGFP (heterozygous) animals was established and then crossed with Id2-CreER; Dlx5/6-Flpe breeders. Genotyping was performed on genomic DNA extracted from pup toe clippings using the following primers (sequences are 5’ to 3’):

- Cre (fwd primer: caacgagttcatgcgcttc, rev primer: cgccgctaacagttgaaaca, product = 304 bp),
- Flpe (fwd primer: tctttaggcagggtagga, rev primer: aagcacgttctggttccc, product = 312 bp),
- Flpo (fwd primer: ccacattcatcaactggtc, rev primer:...
gggccgttcttgatagcgaa, product = 355 bp), and NPY-hrGFP (fwd primer: atgtggacggcagaagatc, rev primer: gtgcggttgcctgactgga, product = 400 bp). To activate CreER, a stock solution of tamoxifen (Sigma T5648; dissolved in corn oil at 20 mg/ml on a shaker at 55°C for several hours) was administered to P21-P35 animals by oral gavage (4 mg/20g mouse) 3-4x over 5-7 days. The efficacy of tamoxifen induction of CreER mediated reporter activation was assessed by histology (see below); well labeled brains typically exhibited >50 labeled cells in a 20 μm thick tissue section of S1BF encompassing 4-5 barrels (~1300 μm x 1700 μm field). All animals were group housed in an SPF barrier facility on a 12 hour light/dark cycle, with unlimited access to food and water. All experiments were performed in accordance with protocols approved by the Department of Comparative Medicine at the NYU Grossman School of Medicine.

Histology

To obtain brain tissue for histology, animals (P40-P60) were transcardially perfused with 4% paraformaldehyde/PBS (diluted from a 32% stock; Electron Microscopy Sciences cat#15714) and the brain dissected, with a post-dissection fix period of 0-16 hrs. For the preparation of thin cryosections, brains were equilibrated in 30% sucrose/PBS overnight before freezing in mounting medium (Tissue-Plus O.C.T. compound; Scigen 4583). For the quantification of Id2 vs non-Id2 interneurons in Figure 1, vibratome sections (50 μm) were generated following perfusion/PBS washes using a Leica vibratome (VT1000S). Brain cryosections (20 μm) were generated using a Leica CM3050 cryostat, collected on glass slides (Shandon ColorFrost Plus; ThermoScientific cat#9991013), and stored at -20°C. For immunohistochemistry (IHC), tissue sections were washed in PBS, blocked for 1 hour at room temperature with PBS/0.1% Triton-X 100/2% normal donkey.
serum, incubated overnight at 4°C with primary antibody (rabbit anti-CCK; Frontier Institute cat#Af350; 1:500 dilution) in blocking solution, washed in PBS, incubated 1 hour at RT with secondary antibodies (for CCK IHC: donkey anti-rabbit AlexaFluor-488; Invitrogen cat#A-21206; 1:1000 dilution). Fluorescence in situ hybridization for NPY and tdTomato (Figure 2A-B) was performed as described previously (33), with the following primers used to amplify templates for labeled cRNA antisense probe synthesis: NPY fwd primer: 5’-tcacagaggacccagagc-3’; NPY rev primer (w/T7 sequence): 5’-attaatacgactcactatagcggagtccagcctagtggtg-3’; tdTomato fwd: 5’-atcaaagagttcatgcgcttc-3’; tdTomato rev primer (w/T7 sequence): 5’-cattaatcagactcataaggttccacgatggtgtagtcctc-3’.

Slice preparation

Adult transgenic mice of either sex (postnatal day range 30-120; mean age = 52 days) were terminally anesthetized with isoflurane, and once unresponsive, were transcardially perfused with ice-cold sucrose-ACSF containing the following (in mM): 87 NaCl, 75 sucrose, 2.5 KCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 10 glucose, 1.0 CaCl₂, and 2.0 MgCl₂, saturated with 95% O₂/5% CO₂. Next, the mice were decapitated and the brains were extracted. The caudal part of the brain was glued to a stage, such that the rostral part of the brain was pitched forward 15°. The stage was then placed into a chamber filled with bubbled ice-cold sucrose ACSF, and the brain was sliced into 300 µm-thick coronal sections using a Leica VT1200S vibratome. The slices were incubated at 35°C for 30 min in the above sucrose solution or the recording-ACSF described below and then transferred to room temperature for at least 1 hour before recording commenced.

Electrophysiology recordings
Slices were transferred to a recording chamber perfused with ACSF containing (in mM): 120 NaCl, 2.5 KCl, 25 NaHCO₃, 1.4 NaH₂PO₄, 21 glucose, 0.4 Na-Ascorbate, 2 Na-Pyruvate, 2 CaCl₂, and 1 MgCl₂, saturated with 95% O₂/5% CO₂ and maintained at 29 – 32°C. For some experiments, the bath solution contained the NMDA receptor blocker D-AP5 (25 µM; Abcam), the AMPA receptor blocker CNQX (10 µM; Abcam), or the GABA<sub>A</sub> receptor blocker CGP-35348 (60 µM; Tocris). Neurons were visualized on an upright Olympus microscope (BX50WI or BX51WI) using DIC and fluorescence illumination from an LED power source (Mightex) for tdTomato and/or GFP. All neurons were recorded in the barrel field of the primary somatosensory cortex in layers 2-5. Layers were identified visually under DIC optics using several features. The L1-2 border was marked by a sharp increase in soma density from L1. The L3-4 border was identified under 4x power by the top of the barrels, the presence of horizontal fibers under 40x (in L4), and the absence of pyramidal cells (PCs) seen in L2/3. L5a was a clear band below L4 and the L5b-6 border was determined under 40x by a sharp drop in PCs. Neurons were recorded in whole-neuron patch-clamp in current clamp and voltage clamp mode using an internal solution containing (in mM): 130 K-Gluconate, 10 HEPES, 1.1 EGTA, 2 Mg-ATP, 0.4 Na-GTP, 10 Na-phosphocreatine, and 1.5 MgCl₂, 0.3-0.5% biocytin, and titrated with 1 M KOH to a final pH of 7.3. Glass pipettes were pulled on a horizontal puller (Sutter Instruments) using borosilicate glass (inner/outer diameter 1.5 mm / 0.86 mm) and had resistances of 2-6 MΩ. Before gaining whole-neuron access, a gigaseal was obtained, and the pipette capacitance was compensated. Access resistances were monitored throughout recordings and were completely compensated. The intrinsic properties of neurons with access resistances >40 MΩ were not analyzed. All data were
collected using a Multiclamp 700B amplifier (Molecular Devices), a Digidata digitizer (1440A or 1550B series, Molecular Devices), and Clampex version (10.6 or 10.7) software (Molecular Devices); data were sampled at 20 kHz and low-pass filtered at 10 kHz.

Electrophysiological characterization and analysis

A total of 178 cells were recorded. This includes a sample of 76 cells that were randomly selected for patching, and this group was used to determine the proportions of each type. In addition, neurons were recorded in the same Id2-CreER; Dlx5/6-Flpe; Ai65 mice but recordings were biased to superficial layer 2/3 to maximize the chances of obtaining non-LS Id2 INs. Lastly, a third group included cells from animals where NPY cells were labeled with GFP (i.e., Id2-CreER; Dlx5/6-Flpe; Ai65; NPY-hrGFP) to facilitate the identification of non-LS Id2 INs. Neurons were characterized for intrinsic and active properties in current-clamp mode from a resting potential of ~-70 mV. Neurons were injected with 1 s long square pulses of increasing current. Parameters relating to the neuron’s intrinsic and active properties were assessed as follows:

First spike latency and cell type classification: Once rheobase was determined, several current steps were delivered at rheobase to characterize the behavior of the cell. The recorded cells fell into three readily separable groups. The first and most numerous group exhibited a single-spike at rheobase with latencies of >500 ms and were termed late-spiking (LS). The second group displayed a high-frequency (>100 Hz) burst of two or more APs at rheobase and were termed burst-spiking (BS). The third group of cells had single spikes at rheobase with short latencies (<100 ms) consistent with the charging of the membrane capacitance (first AP latency normalized to membrane $\tau$, $\tau$,
mean ± SEM: 4.3 ± 0.5 compared to LS: 70.1 ± 4.1). These cells also exhibited irregular spiking (CV of ISI: 0.112 ± 0.029 vs. 0.053 ± 0.004 in LS, p=0.0066, Mann-Whitney Test, see the section below for how CV of ISI was measured) and were classified as irregular-spiking (IS).

*Input resistance:* This was determined from Ohm's Law based on the voltage response to a 150 ms long negative voltage step of typically 20 pA.

*Membrane time constant* ($\tau$): This was determined by fitting an exponential decay to the voltage response from 1 s long current pulses below rheobase.

*AP waveform properties:* To measure these properties, only the first APs from each sweep were considered, as APs in neurons with initial bursts tended to widen significantly for subsequent APs, and all such APs from sweeps with total AP counts <50 were considered. AP threshold and peak were calculated first and then t=0 was defined at the time of AP peak. AP threshold was the voltage reading when the voltage derivative was 20 mV/ms. AP peak amplitude was the maximum voltage reached in the 2 ms after the threshold was passed. AP half-width was the time of the AP when the voltage was above the midpoint between the threshold and peak. The afterhyperpolarization (AHP) was calculated as the difference in voltage between the AP threshold and AP reset (minimum voltage in 10 ms window after AP peak). Finally, AP max and decay slopes were the maximum and minimum values for dV/dt reached in the 10 ms after the threshold was passed. Parameters were calculated separately for each AP and then averaged together for the mean neuronal value.

*Rheobase and firing rates:* Rheobase was determined to be the current step that resulted in a single spike, or for bursting cells, a single burst. The firing rate was
measured for each voltage sweep by counting the number of spikes in the sweep and taking or interpolating the value at 2 x rheobase.

**Spike frequency adaptation:** For LS cells, spike frequency adaptation was measured at 2 x rheobase and near rheobase, during the first depolarization where a spike train was observed throughout the pulse (1.3 x rheobase). For IS and BS cells, the adaptation index was only measured at 2 x rheobase, since near rheobase, firing was often highly irregular. The adaptation index was measured as follows: For each voltage sweep during which the neuron spiked at least six times (five ISIs), an exponential function, \( f_{\text{adapt}}(t) \), was fit to the firing-rate vs. time plot. The adaptation index (AI) for that sweep was defined as \( \text{AI} = f_{\text{adapt}}(1000 \text{ ms}) / f_{\text{adapt}}(100 \text{ ms}) \), and the values at 1.3 x rheobase or 2 x rheobase were taken or interpolated to represent adaptation for the cell.

**Spiking regularity:** For each voltage sweep, all inter-spike intervals (ISIs) from the last 500 ms of the current step were considered. The spiking regularity was measured as the coefficient of variation (CV) for these ISIs, and the reported value was taken from the sweeps at 2 x rheobase.

**DSI – optogenetics and paired recordings**

For optogenetics experiments, L2/3 PCs were identified visually under DIC, patched in whole-cell mode, and then confirmed as PCs by a brief electrophysiological characterization. Blue light (470 nm) pulses (2 ms / >100 mA) were delivered to the slice using TTL-pulses to a Mightex brand LED under 40x power with the PC soma centered. Optically evoked IPSCs were recorded under a voltage clamp at a holding potential of -50 mV with D-AP5 and CNQX present to block glutamatergic transmission and CGP 35348 to block the large GABA\(_B\) component of the current characteristic of NGFCs (40).
Cannabinoid dependence of DSI was assessed by optogenetic experiments carried out in the presence of the CB1 receptor blocker AM251 (10 µM; Abcam).

For paired recordings, Id2 neurons were identified as tdT+ soma in the Id2-CreER; Dlx5/6-Flpe; Ai65 mouse line or tdT+/GFP± somas in the Id2-CreER; Dlx5/6-Flpe; Ai65; NPY-hrGFP cross. The Id2 firing pattern was briefly characterized, and the neuron was tagged as late-spiking (LS), irregular-spiking (IS), or burst-spiking (BS). A nearby PC was also patched (Id2-PC inter-soma distance range 10-130 µm, average distance ± SEM = 53 ± 4), and connectivity to the Id2 cell was assessed under voltage-clamp for both cells by eliciting escape spikes from the Id2 cell and looking for the presence of IPSCs in the PC at a holding voltage of -50 mV. If a connection was not seen, either a new Id2-PC pair was tested, or the PC pipette was withdrawn with the Id2 neuron still held, and another nearby PC was patched and tested. If a connection was identified, then the DSI protocol was run as described in Figure 4.

Morphology
During whole-cell electrophysiological recordings, neurons were held for at least 15 min with an internal solution containing ~0.4 or ~1.0% biocytin. Some slices, especially the ones with neurons held close to 15 min, were retransferred to the incubation chamber to allow for more biocytin diffusion inside the recorded cell. After filling, the slices were fixed with 4% paraformaldehyde/PBS (diluted from a 32% stock; Electron Microscopy Sciences cat#15714) for 1-7 days at 4°C. Fixed slices were then thoroughly washed with PBS and left overnight at 4°C in a 0.4% streptavidin (AlexaFluor 647 conjugate; Invitrogen) solution (498 µl 0.3% Triton X-100 in PBS, 2 µl streptavidin per slice). Slices were then washed in PBS, mounted with Fluoromount-G (Invitrogen) on a glass
microscope slide, and imaged under a 40-63x oil-immersion objective using a confocal microscope (Zeiss). Confocal images were then used to morphologically reconstruct neurons in three dimensions using Neurolucida. In a few cases, cells were juxtacellulary labeled and processed using CUBIC before morphological reconstruction (33).

**Silicon probe implantation and recordings**

Mice (n = 5, 28–35 g, 3–10 months old) were implanted with 64-site silicon probes (NeuroNexus, Cambridge NeuroTech or Diagnostic Biochips) in the PPC (AP 2.0 mm, ML 1.75mm, DL 0.6mm), as described previously (61). Ground and reference wires were implanted in the skull above the cerebellum, and a grounded copper mesh hat was constructed, protecting, and electrically shielding, the probes. Probes were mounted on plastic microdrives that were advanced to layer 6 over the course of 5–8 d after surgery. A 100–200-μm fiber optic was attached to one of the shanks of the silicon probe. After implantation, animals were allowed to recover for at least 1 week and were housed individually under standard conditions (71–73 °F and 40–50% relative humidity) in the animal facility and kept on a 12-h reverse light/dark cycle. We recorded the mice while they slept or walked around freely in the home cage, and the recording session started 1-2 hr after the onset of the dark phase. Electrophysiological data were acquired using an Intan RHD2000 system (Intan Technologies LLC) digitized with a 30 kHz rate. The wide-band signal was downsampled to 1.25 kHz and used as the LFP signal. For optogenetic tagging of specific neuron types, blue laser light (450 nm, Osram) pulses were delivered in the PPC/V1. The maximum light power at the tip of the optic fiber was
1–4 mW. 100-ms light pulses with 70% of the maximum power were delivered (n = 500–1,000 times at each intensity at 400 ± 200-ms random intervals).

**Unit activity analysis**

Spike sorting was performed semi-automatically with KiloSort 1 (https://github.com/cortex-lab/KiloSort), as previously described (61) and using our own pipeline KilosortWrapper (a wrapper for KiloSort, DOI; https://github.com/brendonw1/KilosortWrapper). This was followed by manual adjustment of the waveform clusters using the software Phy 2 (https://github.com/kwikteam/phy) and plugins for phy designed in the laboratory (https://github.com/petersenpeter/phy-plugins). Unit clustering generated three clearly separable groups based on their spike autocorrelograms, waveform characteristics and firing rate (83). Pyramidal cell (PC), narrow waveform (NW) INs and wide waveform interneurons (WW) were tentatively separated based on these two clusters. A more reliable cell identity was assigned after inspection of all features, assisted by monosynaptic excitatory and inhibitory interactions between simultaneously recorded, well-isolated units and light responses (61, 100). Units were defined as optogenetically responsive cells based on the combination of three criteria (https://github.com/valegarman/hippoCookBook): (1) an average firing response higher than 2SD, (2) significant modulation using a p-value cutoff of $10^{-3}$ (45) and (3) against randomly shuffled pulse times (500 replicates) and testing for significant difference between the observed value and the random distribution. To calculate the layer identity of the units, we aligned depth profiles of electrophysiological landmarks. These included the largest amplitude peak of high-frequency LFP power (500 Hz–5 kHz) corresponding...
to mid-layer 5 and four prominent sinks and sources from the averaged down–up
current-source density (CSD) maps for each animal, as previously described (61).
Rank-order estimation was used to identify the position of units repeating consistently
across up events. The timing from multiple units recorded simultaneously was
transformed in a normalized sequence order from 0 to 1 within an up event. The rate
centroid from each unit in an up event was considered for the rank order.
Brain state scoring was performed as previously described (61, 100, 101) with the
Briefly, spectrograms were constructed with a 1-s sliding 10-s window FFT of 1250 Hz
data at log-spaced frequencies between 1 and 100 Hz. Three types of signals were
used for state scoring: broadband LFP, narrowband theta frequency LFP, and
electroymogram (EMG). For broadband LFP signal, principal components analysis
(PCA) was applied to the z-transformed (1-100 Hz) spectrogram. The first principal
components in all cases were based on power in the low (< 20 Hz) frequency range and
had negatively weighted power in the higher (> 32 Hz) frequencies. The state scoring
algorithm was performed by a series of divisions with thresholds set at the trough
between the peaks of distributions in these three metrics. After automated brain state
scoring, all states were manually reviewed by the experimenter. The awake-sleep index
was estimated as \( \frac{\text{rate}^{\text{Awake}} - \text{rate}^{\text{Sleep}}}{\text{rate}^{\text{Awake}} + \text{rate}^{\text{Sleep}}} \), where \text{rate}^{\text{Awake}} is the
average between the firing rate during Run and Quiet, and \text{rate}^{\text{Sleep}} was the average
between REM sleep and NREM sleep.

Statistical analysis
Statistical analyses were performed with standard MATLAB functions or GraphPad Prism. No specific analysis was used to estimate a minimal population sample, but the number of animals, trials, *in vitro* and *in vivo* recorded cells were larger or similar to those employed in previous works (100). All data presented were obtained from experimental replicates with at least four independent experimental repeats for each assay. All attempts of replication were successful. Data collection was not performed blinded to the subject conditions. Data analysis was performed blinded to the scorer or did not require manual scoring. Unless otherwise noted, for all tests, non-parametric two-tailed Wilcoxon’s paired signed-rank test and Kruskal-Wallis one-way analysis of variance were used. For multiple comparisons, Tukey’s honestly significant difference (HSD) test was employed, and the corrected *p < 0.05, **p < 0.01, ***p < 0.001 are indicated. P-values for Spearman’s correlations are computed using a Student’s t distribution for a transformation of the correlation. Results are displayed as median +/- 25th/75th percentiles, unless indicated otherwise. Dispersion represents ± IC95.

**Source data files**

- Figure 1-Source data 1: Id2 vs. non-Id2 cell counts in S1BF
- Figure 2-Source data 1: Id2 NPY CCK cell counts in S1BF
- Figure 4-Source data 1: DSI source data
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Acknowledgements

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Declaration of interests

The authors declare no competing interests.
Figure 1: Id2 expression delineates a fourth major group of INs.

(A) Public scRNAseq data (from Tasic et al., 2016; comprised of ~ 3000 INs purified from the visual cortex, with transcriptome diversity represented as tSNE plots; see Mayer et al., 2018) underlying the hypothesis that the non-VIP CGE-derived IN population can be distinguished by the expression of Id2. Primary IN lineages originating from the medial (MGE) and caudal (CGE) ganglionic eminences express Lhx6 and Adarb2, respectively. To a first approximation, MGE-derived INs are comprised of parvalbumin (Pvalb) and somatostatin (Sst) subtypes, whereas CGE-derived INs consist of vasoactive intestinal peptide (Vip) and inhibitor of DNA binding 2 (Id2) subtypes. Expression of the marker genes Reelin (Reln), neuron-derived neurotrophic factor (Ndnf), neuropeptide Y (Npy) and cholecystokinin (Cck) is evident within the Id2 population, which roughly corresponds to the lysosomal associated membrane protein 5 (Lamp5) plus synuclein-γ (Sncg) IN categories in Tasic et al., 2016.

(B) Intersectional genetic strategies for targeting Id2 INs. The Id2-CreER knock in driver line combined with the Dlx5/6-Flpe transgenic line allows for labeling of Id2 INs with tdTomato when crossed with the Ai65 intersectional reporter (or the channelrhodopsin CatCh when crossed with Ai80). The use of the intersectional/subtractive reporter FLTG enables the dual color labeling of Id2 and non-Id2 INs. (C) Image of a coronal cryosection (20 μm thick) of an Id2-CreER; Dlx5/6-Flpe; Ai65 labeled brain (P30) reveals the distribution of Id2 INs throughout the layers of the cortical S1 barrel field (S1BF). (D) Image of a coronal field in S1BF of Id2-CreER; Dlx5/6-Flpe; FLTG labeled brain with Id2 INs in green and non-Id2 INs in red. (E) Proportion of Id2 vs. non-Id2 INs across the layers of S1BF determined from cell counts of the cross in (D). Id2 INs
comprise 18% of the overall IN population (3812 cells counted across 4 brains; see also Figure 1-Source data 1). (F) Distribution of Id2 and non-Id2 IN somas across S1BF lamina. Scale bars in panels C and D represent 100 μm.
Figure 2: Histological analysis of Id2 INs in S1BF.

(A-B) Assessment of Npy expression in Id2 INs (Id2-CreER; Dlx5/6-Flpe; Ai65).

Fluorescent in situ hybridization (FISH) with cRNA probes for tdTomato (red; panel A) and Npy (green; both red and green channels shown in panel B) mRNAs on brain cryosections (20 μm thick) reveals that outside of L1, the vast majority of Id2 INs express Npy (L2-5: 87%). The percentages of Id2/Npy cells (yellow) in each layer are represented as pie charts on the right. (C-D) Assessment of CCK expression in Id2 INs (Id2-CreER; Dlx5/6-Flpe; Ai65). Immunofluorescent histochemistry (IHC) for tdTomato (red; panel A) and CCK (green) reveals that a fraction (15%) of Id2 cells in L2-6 express high levels of CCK (CCK\textsuperscript{hi}; white arrowheads), with the highest proportion of Id2/CCK cells observed in L2 and L6. The percentages of Id2/CCK cells (yellow) in each layer are represented as pie charts on the right. Scale bars in panels B and D represent 100 μm. See also Figure 2-Source data 1.
Figure 3. Electrophysiology and morphology of Id2 INs in S1BF. (A-D) Four examples of the types of cells encountered in this survey and their respective locations in the cortical column: (A-B) Late-spiking (LS), (C) Irregular-spiking (IS), and (D) Burst-spiking (BS). For electrophysiological characterization, cells were injected with current to bring them to -70 mV. Each voltage trace shows the response of the cell to a 1s long current injection at the indicated levels which are: i) a negative current to hyperpolarize the cell to -100 mV [black-lower trace], ii) current steps just below [grey trace], at [blue trace], and just above rheobase [red trace], and iii) current at roughly double the rheobase [black-upper trace]. To the right of each cell’s voltage traces is the morphological reconstruction from biocytin fills of the same recorded cell with the cortical layers and barrel fields indicated with dashed lines. Dendrites are shown in black and axons are shown in gray. (E) Overlay of first action potential waveforms at rheobase from the three described cell types. (F) Proportions of the three main cell types encountered in L2/3 and L4/5. For L2/3, 45 randomly selected cells were patched and characterized electrophysiologically. The proportions of the cell types were: 80% LS, 18% IS, and 2% BS. For L4/5, 31 randomly selected cells were characterized, and the proportions of the cell types were: 90% LS, 10% IS, and 0% BS. (G) 3D plot of LS, BS, and IS Id2 INs resolved by AP half-width, rheobase, and AP latency.
<table>
<thead>
<tr>
<th>Electrophysiological property</th>
<th>LS in L2/3 (n ≥ 15)</th>
<th>LS in L4-5 (n ≥ 5)</th>
<th>IS in L2/3 (n ≥ 5)</th>
<th>BS in L2/3 (n ≥ 5)</th>
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<tr>
<td>First AP latency (ms)</td>
<td>805 ± 22</td>
<td>827 ± 32</td>
<td>53 ± 4</td>
<td>143 ± 37</td>
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<tr>
<td>Input Resistance (MΩ)</td>
<td>188 ± 7</td>
<td>130 ± 11***</td>
<td>199 ± 32</td>
<td>301 ± 32***</td>
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<td>Membrane τ (ms)</td>
<td>12 ± 0.5</td>
<td>11 ± 0.8</td>
<td>13 ± 1.2</td>
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<td>AP half-width (ms)</td>
<td>0.70 ± 0.02</td>
<td>0.63 ± 0.08</td>
<td>0.48 ± 0.04***</td>
<td>0.57 ± 0.05*</td>
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<td>AP threshold (mV)</td>
<td>-35 ± 1</td>
<td>-33 ± 2</td>
<td>-38 ± 2</td>
<td>-41 ± 2*</td>
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<td>AP rise slope (mV/ms)</td>
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<td>367 ± 61</td>
<td>404 ± 60</td>
<td>322 ± 46</td>
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<tr>
<td>AP fall slope (mV/ms)</td>
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<td>-108 ± 20</td>
<td>-150 ± 19***</td>
<td>-135 ± 29*</td>
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<td>AHP amplitude (mV)</td>
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<td>12.9 ± 1.1</td>
<td>9.9 ± 0.9***</td>
<td>6.6 ± 1.3***</td>
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<td>Rheobase (pA)</td>
<td>139 ± 14</td>
<td>205 ± 44</td>
<td>155 ± 18</td>
<td>51 ± 7***</td>
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<td>Firing rate (Hz) (at 2 x rheobase)</td>
<td>38 ± 3</td>
<td>29 ± 4</td>
<td>40 ± 6</td>
<td>31 ± 7</td>
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<td>Firing Regularity (at 2 x rheobase)</td>
<td>0.053 ± 0.004</td>
<td>0.050 ± 0.008</td>
<td>0.112 ± 0.029**</td>
<td>0.262 ± 0.124**</td>
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<tr>
<td>Adaptation Index (near rheobase)</td>
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<td>1.06 ± 0.11</td>
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<tr>
<td>Adaptation Index (at 2 x rheobase)</td>
<td>0.76 ± 0.03</td>
<td>0.72 ± 0.03</td>
<td>0.26 ± 0.05***</td>
<td>0.24 ± 0.14**</td>
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Table 1. Electrophysiological properties of Id2 INs in S1BF. Values are reported as Mean ± SEM. Significance was tested for each group against LS in L2/3 using the Mann-Whitney test, * denotes significance between 0.01-0.05, ** denotes significance between 0.001-0.01, and *** denotes significance <0.001. Significance was not assessed for the first spike latency as cells were categorized on that basis. For some cells, not all of the parameters were assessed, so the n values are the minimum number of cells used to calculate all of the parameters. See Materials and Methods section for how these parameters were obtained. See also Table 1-Source data 1.
Figure 4. The non-LS Id2 population exhibits DSI. (A) Protocol used for revealing depolarization-induced suppression of inhibition (DSI). DSI was first assessed via optogenetics in L2/3 pyramidal cells (PCs) in the following manner: PCs were recorded in voltage-clamp mode in cortical slices from Id2-CreER; Dlx5/6-Flpe; Ai80 animals, in which the channelrhodopsin CatCh is widely expressed in the Id2 IN population. Control IPSCs were elicited by light pulses of 2ms in duration. Then, after a 60s interval, the PC was stepped to +10 mV for 5s, and after a 2s recovery, another IPSC was elicited in the same manner. (B) Sample traces from optogenetic experiments. In the drug-free condition (left panel), the peak current decreases slightly but noticeably and recovers by the next stim 60s later. However, in the presence of the CB1 receptor antagonist AM251 (10 µM) [right panel], this decrease was not observed. (C) Summary data from all cells tested as in (B). Thin, faint lines are the averages for each cell across three or more repetitions. Dark, solid lines are the averages across all cells. The average decrease in IPSC amplitude in the control condition was 16±2% (n = 18 cells, N = 8 animals). Two-tailed paired t-tests of Post vs. Pre and Recovery vs. Post were significant (p=1e-6, p=6e-6). The average decrease in IPSC amplitude in the AM251 condition was 8±1% (n = 11 cells, N = 4 animals). Also, the average decrease post-depolarization in Control vs. AM251 was significant by a two-tailed unpaired t-test (p=0.002). (D) A subset of five cells was tested in which the DSI protocol was carried out with (dp+) or without (dp-) a depolarization to +10 mV to control for variability or rundown of the synaptic current. Trials that incorporated the depolarization step exhibited significantly greater decreases in IPSC amplitude than those that lacked it (p=0.014, two-tailed paired t-test). (E) DSI was also detected in nonLS-PC pairs but not in LS-PC pairs. Recording configuration
and sample current traces from two PCs: a post-synaptic PC to a nonLS cell (left) and a
post-synaptic PC to an LS cell (right). Current traces represent a DSI experiment.

Several probe stimuli were given to obtain a baseline average (black trace), then the
depolarization step to +10 mV was delivered to the PC, and 1-2 s later, the PC was
probed again (red trace). The cell was then probed several times afterwards to assess
recovery (grey trace). The mouse lines used for these experiments were Id2-CreER;
Dlx5/6-Flpe; Ai65 or the same with the addition of an NPY-hrGFP allele to facilitate the
identification of nonLS cells. Out of 46 nonLS-PC pairs tested, only 9 (20%) were
connected (compared to 24% for CB₁IS→P in Galaretta et al., 2008). Out of 27 LS-PC
pairs tested, 19 (70%) were connected. DSI was assessed in 9 LS-PC pairs and not
observed in any of them, whereas out of 5 nonLS-PC pairs tested for DSI, 3 pairs
exhibited it. See also Figure 4-Source data 1.
Figure 5. Activity of Id2 INs in vivo. (A) Schematic of the opto-tagging experiments that were used to identify Id2 INs in freely behaving mice. Combined light fiber-recording probes were implanted in Id2-CreER; Dlx5/6-Flpe; Ai80 mice. (B) Peristimulus time histogram (PSTH) from 31 isolated neurons from a single recording session. Note one light-responsive neuron in position 11. The peristimulus raster plot, waveform and auto-correlogram for that light-responsive Id2 IN-light responsive unit are shown in brown. All remaining cells from this session are shown in clear gray for comparison. (C) Peristimulus histogram (PSTH) for all isolated units classified as Id2 IN-light responsive neurons (from n = 5 mice). (D) Average spike waveform (mean ± 95% confidence interval (CI95)) and through-to-peak spike duration (inset; p < 10\(^{-61}\), Kruskal-Wallis test) for PCs, NW INs, WW neurons and Id2 INs (n = 317 PCs, 155 NW INs and 88 WW neurons). The average waveform from all Id2 IN-light responsive neurons are overlayed in yellow. (E) Same as in D but for the average firing auto-correlogram (ACG) in log scale and time constant of the rising phase of the ACG (inset; P < 10\(^{-24}\)). (F) Median firing rates of PCs, NW INs, WW neurons, and light responsive-Id2 INs (Cell type: F\(_{3,2261}\) = 151.44, P < 10\(^{-89}\); State: F\(_{3,2261}\) = 10.74, p = 0.035; Interaction: F\(_{9,2261}\) = 0.97, p = 0.46). Sleep to Awake index (p = 0.051, Kruskal-Wallis test) and coefficient of variance of the firing rate (p = 0.034, Kruskal-Wallis test) as insets. Sleep to Awake index was near zero only for the Id2 IN group (PC: Z = 2.55, p = 0.010; NW: Z = 3.16, p = 0.002; WW: Z = 0.08, p = 0.98; one-sample Wilcoxon signed rank test). (G) Peri-down-state Z-scored firing histogram for light-responsive-Id2 INs (top), and all PCs, NW INs, WW neurons (middle to bottom), as ranked according to their magnitude of event response. Solid lines delimit the ±50-ms window used to estimate the unit responses.
during down-states. Dashed lines delimit the +90-ms to +200-ms window used to
estimate the post-down-state responses. Pie charts show the fraction of units exhibiting
an early increase in firing rate during the post-down epoch above 2 standard deviations
(s.d.). (H) Temporal dynamics of the Z-scored peri-down-state responses across all
groups (top; mean ± IC95). Contrary to what was observed in PCs, NW INs and WW
neurons, Id2 neurons significatively increased their firing rate above baseline levels
during the post-down epoch (post-down: p < 10^{-8}; down: p < 10^{-15}). (I) The magnitude of
the post-down-state response as a function of the activity during the down-state events.
Only Id2 neurons show a significant correlation (Spearman correlation). (J) Group
differences of the average rank position during up-state epochs (p < 10^{-5}, Kruskal-Wallis
test). Id2 INs lead the up-state sequences.
Figure 6. Circuit effects of Id2 IN stimulation *in vivo*. (A) Fraction of negatively modulated (neg mod), positively modulated (pos mod), and unmodulated neurons (no mod) in the PC, NW and WW groups across cortical layers. Average peristimulus histogram for all three modes of response (pos mod, neg mod, and no mod) is shown in the top inset. (B-C) Peristimulus histograms for PCs, NW INs, and WW neurons, each separated into 5 quantiles based on their +0-ms to +100-ms average firing rate response (light to dark shades). Changes in firing rates are shown as s.d. in (B) and Hz (log10 scale) in (C). (D) Peak latency for the light-evoked responses in all neuron groups as a function of the +0-ms to +100-ms rate response. Positively modulated responses peaked consistently later than negative responses (Spearman correlation).
Figure 1 – figure supplement 1: IN scRNAseq gene expression heat maps. (A)

Annotated dendrogram and gene expression heat maps based on combined cortical and hippocampal IN scRNAseq data published by the Allen Institute (Yao et al. 2021; portal.brain-map.org/atlases-and-data/rnaseq). Heat maps illustrate mRNA expression levels (trimmed mean (25%-75%) LOG2(CPM+1)) for each gene listed: Lhx6 (pan-MGE), Adarb2 (pan-CGE), Vip, Id2, Npy, Reln, Ndnf, Lamp5, Cck, and Sncg. Based on our interpretation of these and other gene expression patterns, we added assignments beneath the major branches of the dendrogram to indicate the putative IN subtypes that correspond with each set of color-coded bins. The annotated dendrogram has been adapted from Figure 2A from Yao et al., 2021. The heat maps are reproduced from the Allen Institute for Brain Science, Allen Brain Map, available from portal.brain-map.org/atlases-and-data/rnaseq, scRNAseq portal https://celltypes.brain-map.org/rnaseq/mouse_ctx-hpf_10x. Copyright Allen Institute for Brain Science 2023. All rights reserved. Further reproduction of this panel would need permission from the copyright holder. (B) Pie chart of the nine IN bins (numbered in (A)) where trimmed mean Id2 mRNA levels are >300 counts per million illustrates the main populations in L2-6 that are expected to be labeled in the Id2-CreER; Dlx5/6-Flpe; Ai65 intersectional cross. NDNF and α 7 bins were excluded since these cells are primarily located in L1. Cells in the NGFC (MGE) branch (i.e., Lamp5/Lhx6) and NtnG1 HPF branch were also excluded since these cells are almost entirely located in the hippocampus. The number of cells in each bin was obtained from the Allen scRNAseq data portal (portal.brain-map.org/atlases-and-data/rnaseq) and includes cells isolated from throughout the cortex and hippocampus. (C) Plots of expression levels (counts per million) of Cck,
Sncg, Npy, and Id2 transcripts across the nine Id2 IN bins shown in (B). Cck exhibits variable levels of expression across the non-NGFC bins, with the highest levels in bin 35. Sncg is weakly expressed or absent in all but bin 35. Bin 12 (NGFC) shows the highest Npy level, as expected. See also Figure 1- figure supplement 1 - Source data 1.

**Figure 1 – figure supplement 2: Prevalence and distribution of Id2 INs in prelimbic cortex.** (A) Representative image of dual color labeling of Id2 INs (green) and non-Id2 INs (red) in the mPFC of the Id2-CreER; Dlx5/6-Flpe; FLTG cross. The prelimbic area (PrL) is denoted by the white dotted lines. The scale bar indicates 100 μm. (B) Quantification of the distribution and proportion of Id2 INs in PrL. Due to the difficulty in identifying precise layer boundaries in PrL, green and red cells were counted across 20 equally spaced bins across the pia to the white matter (438/2424 cells counted; n=3 brains; error bars indicate SEM). The overall fraction of Id2 INs/total INs was 18% (represented as a pie chart). See also Figure 1 – figure supplement 2 - Source data 1.
**Figure 1 – figure supplement 3: Id2 INs show little overlap with Nkx2.1-lineage or VIP cells.** (A) Tissue section of the cortical column in S1BF of an Id2-CreER; Nkx2.1-Flpo; Ai65 brain. Arrows point to the two labeled cells in this field, both located in L6. (B) Tissue section of the cortical column in S1BF of an Id2-CreER; VIP-Flpo; Ai65 brain. The white arrow indicates the one labeled cell in this field, located in upper L2/3. Cell labeling was extremely sparse in both crosses. (C) Tissue section of the mPFC of an Id2-CreER; Nkx2.1-Flpo; Ai65 brain. Arrows point to the two labeled cells in this field. (D) Tissue section of the mPFC of an Id2-CreER; VIP-Flpo; Ai65 brain. The white arrows indicate the labeled cells in this field, mostly located along the L1/L2 border. DAPI counterstain (blue) is present in all images. All tissue sections are 20 μm thick. Scale bars indicate 100 μm.
**Figure 1 – figure supplement 4: Methodological considerations for assessing gene expression (the cautionary tale of CCK).** (A) Expression levels of *Cck* mRNA across all GABAergic INs from Tasic et al., 2016 (~3000 INs individually purified from V1), represented on a log scale. The top bar shows the IN subtype categories described in Tasic et al., 2016, with a heat map for *Cck* mRNA shown underneath. On the right, a schematic depiction of the hypothetical extent of cell labeling using immunohistochemistry (IHC), *in situ* hybridization (ISH), or CCK-Cre cumulative reporter labeling (e.g., Cck-Cre; Ai14) is shown. (B) Dual fluorescence *in situ* hybridization (FISH) for *Cck* (red) and *Pvalb* (green) on a brain tissue section (S1BF; 20 μm thick) does not indicate substantial overlap between these markers. However, as shown in (C), the use of CCK-Cre with a tdTomato reporter (in this case, CCK-Cre; Dlx5/6-Flpe; Ai65) in combination with IHC for PV (green) results in substantial labeling of PV INs (white arrows). (D) CCK-Cre cumulative recombination can also label putative NGFC (NPY+), as shown in an intersectional CCK-Cre; Htr3a-Flpo; Ai65; NPY-hrGFP cross (white arrows indicate tdTomato/NPY+ cells). Scale bars indicate 100 μm.
Figure 1 – figure supplement 5: Comparison of 5HT3aR lines in S1BF. (A) Images of cell labeling in S1BF obtained with one copy of Htr3a-Flpo (het: Htr3a-Flpo/+/Ai65F/+)) or two copies (hom: Htr3a-Flpo/Htr3a-Flpo; Ai65F/+), compared with Htr3a(BAC)-Cre; Ai9 and Htr3a(BAC)-EGFP. Dashed lines indicate approximate layer boundaries. (B) The density of cell labeling (cells/mm²) obtained from the four Htr3a transgenic labeling strategies across all layers in S1BF. (C) The comparative density of cell labeling obtained from the four Htr3a transgenic labeling strategies across all layers in S1BF, normalized to Htr3a(BAC)-EGFP (100%). Overall, one copy of Htr3a-Flpo yielded about 30% of the cell labeling as that seen in Htr3a(BAC)-EGFP animals, with two copies of Htr3a-Flpo doubling this labeling. This observation is consistent with a broad but transient expression of Htr3a mRNA in most CGE INs during embryonic development. The ephemeral nature of Htr3a expression in many CGE INs limits the efficiency of labeling with Htr3a-Flpo, although this can be partially compensated for with two copies of the driver. The Htr3a(BAC)-Cre driver in combination with Ai9 labels ~80% of the cells of the Htr3a(BAC)-EGFP line. Scale bars indicate 100 μm.
Figure 2 – figure supplement 1: Id2 INs and NPY expression in S1BF, V1, and A1.

Examples of Id2 IN labeling and NPY expression (tdTomato and NPY-hrGFP expression in Id2-CreER; Dlx5/6-Flpe; Ai65; NPY-hrGFP animals) in brain tissue sections from S1BF, V1 and A1. Id2 INs labeled with tdTomato are shown on the left panels, with the NPY-hrGFP signal included in the right panels from each cortical area. Red cells indicate Id2 INs without NPY expression, and yellow cells indicate Id2/NPY INs. The approximate locations of layer boundaries are labeled on the right. DAPI counterstain (blue) is present in all images. All tissue sections are 20 μm thick. Scale bars indicate 100 μm.
Figure 3 – figure supplement 1: Additional morphologies of Id2 INs. (A)
Reconstructions of three additional Id2 late-spiking (LS) INs and one Id2 α7 IN in L2-4 of S1BF. Approximate layer boundaries and barrel locations are indicated by dashed lines. (B) Images of ten filled Id2 LS INs aligned to their respective laminar locations. (C) Images of four filled Id2 irregular spiking (IS) INs and pyramidal cell recorded pairs located in L2/3. (D) Images of three filled Id2 burst spiking (BS) INs and pyramidal cell recorded pairs located in L2/3. (E) Image of an α7 IN and pyramidal cell recorded pair. Arrows in (C-E) indicate cell soma of the respective filled Id2 cells. Note: neuronal images are better appreciated by zooming in (especially axonal arbors).
Legends for source data files

Figure 1-Source data 1: Id2 vs. non-Id2 cell counts in S1BF. Counts of Id2 (green) and non-Id2 (red) cells from pia to white matter in Id2-CreER; Dlx5/6-Flpe; FLTG S1BF sections (n=4 brains). Each of the 20 bins used for counting were assigned to 6 layers based on the corresponding depth for the pie charts presented in the main figure.

Figure 2-Source data 1: Id2 NPY CCK cell counts in S1BF. To evaluate expression of NPY in Id2 cells, double FISH (NPY mRNA in green; tdTomato mRNA in red) was performed on thin tissue sections (20 μm) from Id2-CreER; Dlx5/6-Flpe; Ai65 brains (n=3). For cell counting, 11 sections were selected that exhibited both robust and consistent FISH signal across the S1BF area. To evaluate expression of CCK in Id2 cells, IHC for CCK was performed on thin tissue sections from Id2-CreER; Dlx5/6-Flpe; Ai65 brains (n=3). DAPI staining was included for assignment of layer identity in both staining experiments.

Figure 4-Source data 1: DSI source data. This excel file consists of three workbooks, “Fig. 4C (opto; AM251)”, “Fig. 4D (opto; dp +-)”, and “Fig. 4E (paired recordings).” “dp” refers to the 5s depolarization step to +10mV delivered to the PC to induce DSI. Cells discarded from the final data set are not listed. For optogenetics experiments, traces were discarded if the series resistance was too high (>40 MΩ), or if the holding current was too negative (<-100 pA). Cells were discarded if there were fewer than three acceptable traces, if the cell patched was clearly not a PC, or if the recording parameters deviated from the standard protocol (e.g. non-standard internal, interstim interval not 60 s, etc.).

Table 1-Source data 1: Electrophysiological parameters source data. This excel spreadsheet lists all of the cells patched alongside their electrophysiological parameters. The file consists of two workbooks, ‘tabulation’ containing the parameters for each cell, and ‘summary’ containing the summary data (average, sem, n) for each cell type. For some cells, no values are reported for some parameters and these cells are marked ‘n/a.’ A small number of cells were patched with an internal solution different than that reported in the methods. Electrophysiology parameters for these cells were not reported, as differences in internal solution can have significant impacts on electrophysiological parameters, but the cells were counted alongside the rest for the proportions reported in Figure 3. These cells were marked as ‘0’ in the ‘Internal’ column or ‘1’ otherwise. Several cells categorized as LS do not have a value for the AP latency as for these cells a voltage trace bearing a single AP was not obtained. Nevertheless, these cells were confidently labeled LS as the late-onset of the APs is evident even in multiple AP traces. Cells patched in slices with NPY-GFP (i.e., Id2-CreER; Dlx5/6-Flpe; Ai65; NPY-hrGFP) are not listed in this file as the parameters summarized in Table 1 were only taken from the unbiased cross (i.e., Id2-CreER; Dlx5/6-Flpe; Ai65).

Figure 1 – figure supplement 1 - Source data 1: mRNA counts in Id2 cells. Transcriptome data represented as trimmed mean (25%-75%) LOG2(CPM+1) from the Allen Institute (portal.brain-map.org/atlas-and-data/rnaseq) was used to calculate raw
mRNA levels (cpm) from each Id2 bin (Id2 mRNA levels > 300 cpm) for the genes indicated. The number of cells in each bin from their analysis was used to generate the pie chart in the figure. The main NGFC bin (bin 12) is highlighted in the excel file.

Figure 1 – figure supplement 2 - Source data 1: Id2 vs. non-Id2 cell counts in PrL. Counts of Id2 (green) and non-Id2 (red) cells from pia to white matter in Id2-CreER; Dlx5/6-Flpe; FLTG PrL sections (n=4 brains), across 20 evenly spaced bins.
References


Figure 1: Id2 expression delineates a fourth major group of INs

A. Primary lineages, four major groups, Id2 related markers, Id2/Sncg type

B. Diagram of experimental setup

C. Imaging of Id2 expression

D. Additional imaging

E. Percentage of Id2/total INs:
   - L1: 83%
   - L2/3: 27%
   - L4: 8%
   - L5: 7%
   - L6: 15%

F. Laminar position of Id2 and non-Id2 INs
Figure 2: Histological analysis of Id2 INs (S1BF)

% Id2/NPY
L1: 29%
L2/3: 82%
L4: 95%
L5: 90%
L6: 61%

% Id2/CCK<sup>hi</sup>
L1: 2%
L2/3: 14%
L4: 6%
L5: 11%
L6: 25%
Figure 3: Electrophysiology and morphology of Id2 INs in S1BF

A

LS (L2/3)

248 pA

20 mV

200 ms

123, 123, 132 pA

-400 pA

B

LS (L5a)

556 pA

100 μm

L1

L2/3

L4

L5a

238, 240, 242 pA

-750 pA

C

IS (L2/3)

280 pA

140, 140, 188 pA

-200 pA

D

BS (L2/3)

100 pA

L1

L2/3

L4

L5a

40, 42, 44 pA

-50 pA

E

Late-spiking (LS)

Irregular-spiking (IS)

Burst-spiking (BS)

1 ms

F

L2/3

L4/5

G

AP half-width

Rheobase

AP latency

LS

BS

IS
Figure 4: The non-LS Id2 population exhibits DSI

A

Schematic of opto-DSI protocol

B

Control

AM251

Pre-depolarization Post-depolarization Recovery

C

Pre Recovery Post

60 80 100 120 Control

IPSC (% control)

D

dp+ dp-

Pre Recovery Post

60 80 100 AM251

IPSC (% control)

E

Pre-depolarization Post-depolarization Recovery

VC

Id2-IN PC

5 pA 10 ms

5 pA 20 ms

nonLS-PC LS-PC
Figure 5: Activity of Id2 INs in vivo

A

B

C

D

E

F

G

H

I

J

Figure 5: Activity of Id2 INs in vivo

A

B

C

D

E

F

G

H

I

J
Figure 6: Circuit effects of Id2 IN stimulation *in vivo*
Figure 1- figure supplement 1: scRNAseq gene heat maps

A

branch assignment:

2° marker:

Id2 bins in (B):

cell counts:

Lhx6
Adarb2
Vip
Id2
Npy
Reln
Ndnf
Lamp5
Cck
Sncg

NGFC (MGE)
NGFC (GGE)
α7
NDNF
Cxcl14
α7
NtnG1
HPF

B

Id2 bins (Id2>300 cpm):

NGFC (bin 12)

C

Cck levels (cpm)
Sncg levels (cpm)
Npy levels (cpm)
Id2 levels (cpm)
Figure 1 - figure supplement 2: Prevalence and distribution of Id2 INs in prelimbic cortex

A  Id2-CreER; Dlx5/6-Floxed; FLTG

B  Distribution and proportion of Id2 INs

Id2/total INs: 18%
Figure 1 - figure supplement 3: Id2 INs show little overlap with Nkx2.1-lineage or VIP cells

A
Id2-CreER; Nkx2.1-Flpo; Ai65

B
Id2-CreER; VIP-Flpo; Ai65

C
Id2-CreER; Nkx2.1-Flpo; Ai65

D
Id2-CreER; VIP-Flpo; Ai65
Figure 1 - figure supplement 4: Methodological considerations for assessing gene expression (the cautionary tale of CCK)

A

B

C

D

ISH: CCK PV
CCK-Cre; Dlx-Flp; Ai65
CCK-Cre; Htr3a-Flp; Ai65

L1
L2/3
L4
L5a
L5b
L6
wm
Figure 1 - figure supplement 5: Comparison of 5HT3aR lines in S1BF

A

Htr3a-Flpo; Ai65F
het hom

Htr3a(BAC)-Cre; Ai9

Htr3a(BAC)-EGFP

B

Cell density (cells/mm²)

Htr3a-Flpo (het)
Htr3a-Flpo (hom)
Htr3a(BAC)-Cre
Htr3a(BAC)-EGFP

S1BF layer

C

% cell density/Htr3a(BAC)-EGFP

S1BF layer
Figure 2 - figure supplement 1: Id2 INs and NPY expression in S1BF, V1, and A1

S1BF

V1

A1

Id2 INs (Ai65)

Id2 INs (Ai65)

Id2 INs (Ai65)
Figure 3 - figure supplement 1: Additional morphologies of Id2 INs

A

Pia L1
L2/3
L4
L5a
L5b
L6
WM

B

Pia L1
L2/3
L4
L5a
L5b
L6
WM

C

Pia L1
L2/3
L4
L5a
L5b

D

BS

E

α7