

Integration of electrophysiological recordings with single-cell RNA-seq data identifies neuronal subtypes

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Traditionally, neuroscientists have defined the identity of neurons by the cells' location, morphology, connectivity and excitability. However, the direct relationship between these parameters and the molecular phenotypes has remained largely unexplored. Here, we present a method for obtaining full transcriptome data from single neocortical pyramidal cells and interneurons after whole-cell patch-clamp recordings in mouse brain slices. In our approach, termed Patch-seq, a patch-clamp stimulus protocol is followed by the aspiration of the entire somatic compartment into the recording pipette, reverse transcription of RNA including addition of unique molecular identifiers, cDNA amplification, Illumina library preparation and sequencing. We show that Patch-seq reveals a close link between electrophysiological characteristics, responses to acute chemical challenges and RNA expression of neurotransmitter receptors and channels. Moreover, it distinguishes neuronal subpopulations that correspond to both well-established and, to our knowledge, hitherto undescribed neuronal subtypes. Our findings demonstrate the ability of Patch-seq to precisely map neuronal subtypes and predict their network contributions in the brain.

The morphology, excitability, connectivity and neurotransmitter utilization of individual neurons underlie the distinct computations each neuronal circuit can perform in the nervous system^{1–3}. Thus, the identification of distinct subclasses of neurons remains a key challenge in neuroscience. Neuronal taxonomy based on a combination of developmental, morphological and neurophysiological traits is well accepted, particularly for interneurons in the cerebral cortex^{4–6}. These classification systems primarily rely on candidate marker analysis by a mixture of patch-clamp electrophysiology and single-cell semiquantitative PCR (qPCR)^{4,7–10}. More recently, advances in single-cell RNA sequencing (RNA-seq) in the central nervous system^{11–13} led to the identification of novel cell types. Particularly, RNA-seq allowed the molecular classification of neurons in the somatosensory cortex and CA1 subfield of the hippocampus into 47 subtypes, including 16 subclasses of interneurons¹¹. Despite pioneering work using microarrays^{14–16}, multiplexed qPCR^{17,18} and even proof-of-concept RNA-seq on single neurons¹⁹, no robust method exists to simultaneously investigate the electrophysiology, morphology and transcriptome profiles of the same neuron. Combining patch-clamp electrophysiology and *post hoc* morphological reconstructions with the resolution of quantitative RNA-seq in single neurons would present a potentially critical advance for neuronal classification as it can resolve transcriptome-wide variations in gene expression to reveal cell type-specific determinants of neuronal cytoarchitecture and biophysical properties. Nevertheless, only Qiu *et al.*¹⁹ have attempted RNA-seq on material taken from three neurons through a patch pipette without prior patch-clamp recordings, which yielded RNA-seq data of variable quality.

Here, we describe Patch-seq, a method relying on sequencing RNA aspirated from the soma of single patch-clamp-recorded neurons. We validate Patch-seq transcriptomes by aligning them with larger single-cell data sets to achieve high-quality classification, particularly to resolve cortical interneuron types previously considered homogenous into distinct subtypes. We also show that Patch-seq is compatible with the *post hoc* morphological analysis of neurons in optically cleared tissues, and produces a quantitative data set that simultaneously resolves mRNAs for all known ion channels, receptors and synaptic proteins. Through acute pharmacological probing of cortical interneurons, we established causality between RNA-seq-based predictions and experimentally observed neuronal responses *ex vivo*. Thus, Patch-seq is suited to discover molecular determinants of neuronal morphology and excitability.

RESULTS

Data collection and characterization

We focused on cholecystokinin (CCK)-containing(+) GABAergic interneurons (Fig. 1) because their morphological and molecular features are thought to form a quasi-continuum from axon-targeting to dendrite-targeting interneurons in cortical areas^{3,5,11}. CCK⁺ interneurons are the plastic and dynamic gatekeepers of neuronal circuits²⁰. Their inactivation likely contributes to anxiety, mood disorders and schizophrenia^{21,22}. As reliable histochemical detection of CCK⁺ interneurons is particularly challenging, we established a dual-labeled CCK^{BAC/dsRed}::GAD67^{gfp/+} mouse reporter^{23,24} (Fig. 1a and Supplementary Fig. 1a), and sampled dual DsRed⁺/GFP⁺

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interneurons in layers (L)1/2 of the somatosensory cortex. We took advantage of moderate CCK expression in cortical pyramidal cells¹¹ (DsRed⁺ only) to build a reference database of electrophysiological and molecular features that resolve cortical layer specificity for comparative analysis (Fig. 1a).

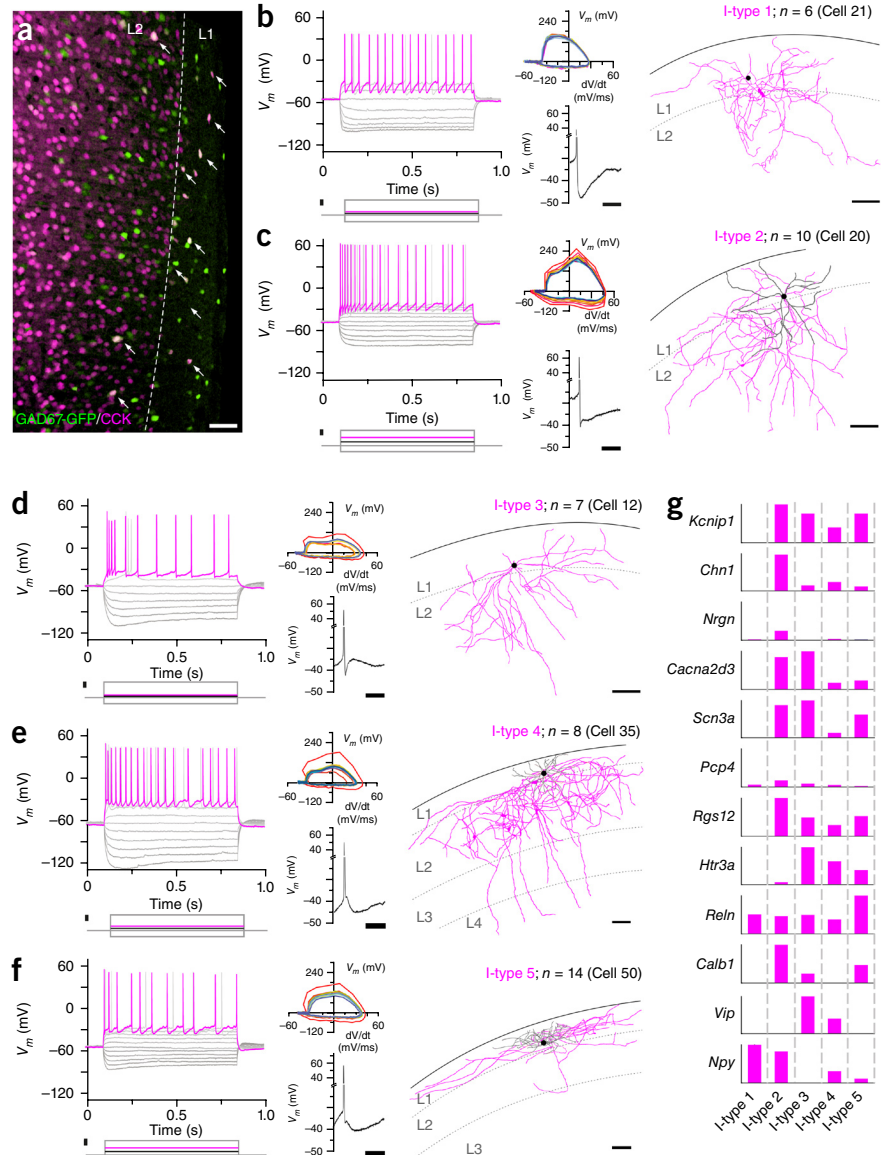
We first selected ~120 DsRed⁺ cortical neurons in total for patch-clamp electrophysiology and morphological examination (as confirmed by epifluorescence microscopy before recording; Fig. 1a), of which 83 cells proved suitable for combined electrophysiology and RNA-seq analysis. Forty-five were interneurons (inhibitory types, “I-types”; Fig. 1b–f) and DsRed⁺/GFP⁺ with an additional 38 being pyramidal cells (excitatory types, “Exc-types”; Supplementary Fig. 1b–d). We discarded neurons only if their input resistance showed >20% deviation from baseline (Online Methods) or if their electrophysiology parameters did not resemble any I-type cluster (three cells; data not shown). For each neuron, we recorded its location (cortical layer) and analyzed its passive and active membrane properties through a series of custom-written routines for whole-cell patch-clamp electrophysiology (Supplementary Table 1). We also combined patch-clamp electrophysiology, biocytin filling, chromogenic (3,3′-diaminobenzidine (DAB)) labeling and tissue clearing for the reconstruction of (axonal) morphology in optically cleared tissues^{25,26} (Fig. 1b–f). Our visualization method is also compatible with lightsheet²⁷ or two-photon microscopy in whole-brain

slices (300- to 350- μm thick), thus greatly reducing processing time and increasing the three-dimensional integrity of neuronal morphology.

Functional and morphological analysis of CCK interneurons

The most common nomenclature for interneurons⁶ combines key biophysical and morphological features. Based on their pattern of postsynaptic target innervation, interneurons fall into one of three morphologically distinct subclasses: axo-axonic, perisomatic or dendrite-targeting cells^{3,5–7,28}. Here, we first used patch-clamp electrophysiology to classify DsRed⁺/GFP⁺ interneurons in cortical L1 into five subclasses (I-types 1–5, Fig. 1b–f and Supplementary Table 1) purely based on their electrophysiological properties. I-type 1 interneurons exhibited action potential (AP) accommodation with a low adaptation ratio. They began to spike at the onset of a rheobasic stimulus without producing a burst, demonstrated the smallest AP amplitude, the largest after-hyperpolarization (AHP) and most-hyperpolarized resting membrane potential (V_{rest} ; Fig. 1b). I-type 2 cells had accommodating AP trains without producing burst firing. These cells displayed the highest AP amplitude, the steepest AP upstroke slope and the highest firing frequency of all recorded neurons.

Figure 1 Neurophysiological diversity, distribution and representative molecular marks of CCK interneurons. (a) Confocal photomicrograph of DsRed/GFP dual-labeled neurons (arrows) in layer L1 of the somatosensory cortex of a CCK^{BAC/dsRed}; GAD67^{GFP/+} mouse (Supplementary Fig. 1a). Scale bar, 50 μm . (b–f) Representative current-clamp recordings of dual-tagged CCK^{DsRed}/GAD67^{GFP} interneurons (I-type 1 (b), 2 (c), 3 (d), 4 (e), 5 (f)). At the left of each panel, AP responses (top) to square current pulses (bottom) are shown. Phase-plane plots of the APs rising from 2 \times rheobase current injection (top right) and rheobasic APs (bottom right) are depicted for every neuronal subtype. In phase-plane plots, the first AP is red and subsequent APs shift from warm to cool blue color. For the rheobasic AP, the y axis between –20 mV to +30 mV was omitted to emphasize AHP and ADP characteristics. Vertical scale bars, 200 pA, horizontal scale bars, 25 ms. To the right of each panel, morphological reconstruction of a representative biocytin-filled interneuron for each subclass is shown. Scale bars, 100 μm . Axons are in red, dendrites in gray. V_m , membrane potential. (g) Cell type-specific expression of a voltage-gated K⁺-channel interacting protein (*Kcnp1*), a GTPase-activating protein (*Chn1*), a protein kinase C substrate (*Nrgn*), a Ca²⁺ channel subunit (*Cacna2d3*), a Na⁺ channel subunit (*Scn3a*), Purkinje cell protein 4 (*Pcp4*), a G protein-signaling regulator (*Rgs12*), serotonin receptor subtype 3a (*Htr3a*), reelin (*Reln*), a superficial layer-specific marker, calbindin D28k (*Calb1*), a Ca²⁺-binding protein, vasoactive intestinal polypeptide (*Vip*) and neuropeptide Y (*Npy*) in subclassified I-type CCK⁺ interneurons.



They did not show sag depolarization (a phenomenon indicating the activation of hyperpolarization-activated nonselective cationic currents; **Fig. 1c**). I-type 3 interneurons produced AP bursts, the shallowest AP upstroke slope and considerable accommodation (**Fig. 1d**). Each AP burst consisted of 3–4 spikes after the first AP on 2-times threshold current. These neurons had a large after-depolarization (ADP), as well as the largest sag depolarization. I-type 4 interneurons were accommodating cells that displayed a small ADP before a slow AHP; yet this was insufficient to produce a burst (**Fig. 1e**). These neurons had the highest input resistance among all interneuron subclasses examined. I-type 5 interneurons exhibited irregular spiking, and fired APs without producing a burst (**Fig. 1f**). These cells had a small-amplitude ADP on the falling trajectory of the AHP rise, and had the highest rheobase among the interneuron types assessed.

For comparison and validation, we examined the 38 pyramidal cells recorded in L2/3, L4 and L5 (Exc L2/3, L4 and L5; **Supplementary Fig. 1b–d** and **Supplementary Table 1**). L2/3 and L4 pyramidal cells (DsRed only) were categorized as nonbursting, accommodating and regular spiking (**Supplementary Fig. 1b,c**). In contrast, L5 pyramidal cells exhibited higher input resistance and pronounced ADP and a second consecutive AP when stimulated at 2× the threshold current (**Supplementary Fig. 1d**).

Next, we used *post hoc* morphological reconstructions to identify the axonal nests and dendritic arbors of the five putative interneuron subtypes (**Fig. 1b–f**). Our analysis revealed substantial differences in cell morphology, such as: I-type 1 interneurons had equally sized processes throughout, thus precluding their unequivocal assignment as axons or dendrites by light microscopy. These processes concentrated in L1 and L2. I-type 1 cells maintained gap junctions with neighboring nonpyramidal cells and even to L2/3 pyramidal cells, as shown by dye-loading experiments using Lucifer yellow (see below). I-type 2 interneurons had somatic diameters at least twice that of I-type 1 cells, and resided at the L1–L2 transition. Whereas their dendrites intruded into both L1 and L2, their axons were almost exclusively located in L2. I-type 3 interneurons, similar to I-type 1 cells, also had processes with homogenous diameters, yet all were less than half of the process thickness of I-type 1 cells. Their axonal arbor was less branched with the majority of processes coursing in L2. I-type 4 cells had the majority of their elaborate axonal nests concentrated in L2 with some collaterals reaching as far as L4. Their dendrites targeted apically. I-type 5 interneurons branched horizontally in L1 and upper L2. Rarely, we saw dye coupling between I-type 5 interneurons and neighboring pyramidal cells, with less dense network labeling than for I-type 1 cells. Cumulatively, the above correlated electrophysiology and morphological differences identify I-type 1–5 CCK⁺ interneurons as bona fide interneuron subtypes. Their distinct features suggest a critical diversity of molecular determinants for cell identity, which can be interrogated by single-cell RNA-seq.

RNA sequencing of somatic aspirates

We next developed a method, called Patch-seq, for performing single-cell RNA sequencing on the same neurons that we characterized by

patch-clamp electrophysiology (**Figs. 2** and **3a–c**). After testing the neurons in a series of current-clamp and voltage-clamp protocols within 20–25 min, their entire somatic compartment was aspirated into the recording pipette. We found, using an iterative approach on ~140 cells (**Supplementary Fig. 2**), that applying a continuum of positive voltage pulses (to membrane potential +20 mV from holding potential –5 mV, each 5 ms in length, at 5-ms intervals) reduced the loss of RNA by most efficiently holding negatively charged RNA molecules in the pipette solution. Subsequently, the samples (0.8–0.9 μ l) were ejected into lysis buffer (0.6 μ l). RNA-seq was performed using a modified version of single-cell-tagged reverse transcription (STRT-C1)^{29,30} designed to handle low RNA input samples in a tube reaction.

Next, we subjected each sample to RNA-seq, generating 1.6 million raw reads per cell, of which 40% mapped uniquely to 2,068 distinct genes (on average, using UCSC Genome Browser conservative gene models, mainly protein-coding ones). On average, we observed 5,977 and 6,760 mRNA molecules in excitatory and inhibitory neurons, respectively (**Fig. 3d**). This corresponds to an absolute capture efficiency of 7% per mRNA molecule per cell, as inferred by comparison with previously published single-cell data¹¹. By comparison, RNA-seq of single neurons from the mouse neocortex can recover ~19,000 RNA molecules/neuron, mapped to ~5,000 distinct genes¹¹, with a capture efficiency of ~20%. We attribute losses to procedural differences, leakage during cell aspiration and/or binding of aspirated RNA to glass or plastic surfaces. Pan-neuronal markers¹¹ *Thy1* and *Stmn2* were detected in 79% and 87% of the cells, respectively.

To validate the quality of our RNA-seq data, we compared the results from inhibitory and excitatory neurons. As expected, I-type neurons expressed *Gad1* (the gene encoding GAD67; 41 of 45 cells) and *Gad2* (the gene encoding GAD65; 39 of 45 cells), as well as *Cck* (44 of 45 cells). Moreover, all subtypes of interneurons contained mRNAs for the vesicular inhibitory amino acid transporter (*Slc32a1*)³¹ but lacked either vesicular glutamate transporter 1 (*Slc17a7*) or 2 (*Slc17a6*) (refs. 32,33). Subsets of these interneurons also expressed mRNAs for neuropeptides (*Vip*, *Npy* and *Crh*) and Ca²⁺-binding proteins (*Calb1*; **Figs. 1g** and **3e**),

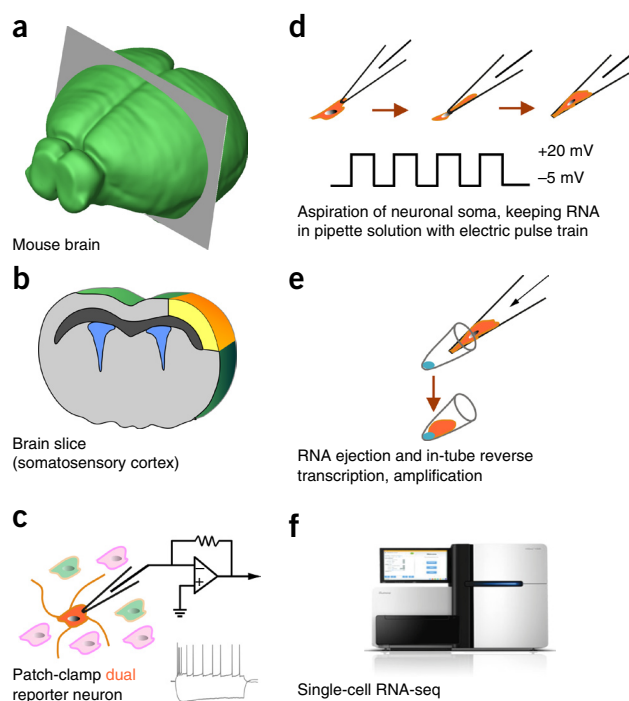
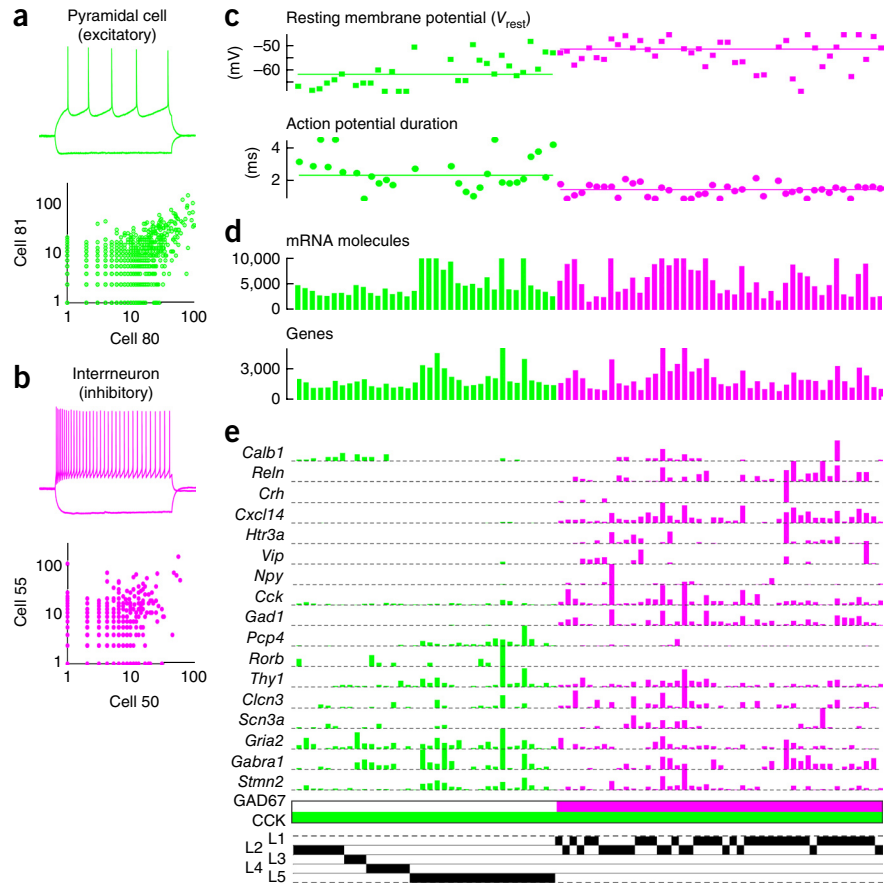


Figure 2 Workflow diagram of Patch-seq procedures. **(a)** Coronal cutting plane of a mouse brain to access the somatosensory cortex. **(b)** *Ex vivo* brain slice anatomy with the somatosensory cortex highlighted in yellow and orange. **(c)** Whole-cell patch-clamp recording of DsRed⁺/GFP⁺ dual-tagged interneurons. **(d)** Aspiration of neuronal somata was followed by square voltage pulses from –5 mV (holding potential) to +20 mV, while maintaining negative pressure. **(e)** The sample was expelled into lysis buffer, which allowed for in-tube reverse transcription by PCR. **(f)** Single-cell RNA sequencing performed on an Illumina HiSeq2000 instrument.

Figure 3 Overview of Patch-seq methodology. (a,b) Analysis of patch-clamp recordings from pyramidal cells and interneurons using large sets of parameters for neuronal classification. Scatter plots between pairs of excitatory and inhibitory neurons reveal high reproducibility. (c) Resting membrane potential (V_{rest} , top) and AP duration (bottom) are shown as examples that, among many others, distinguish (excitatory) pyramidal cells and (inhibitory) interneurons. (d) Bar plots showing the total number of mRNA molecules (top) and the corresponding number of detected genes (bottom) for each cell in the data set. (e) Bar plots showing mRNA molecule counts per cell for select genes at specified anatomical positions. Differential gene expression and layer (L) positioning by pyramidal cells (green) and interneurons (magenta). *Thy1* and *Stmn2* are pan-neuronal markers, *Gad1* is a pan-interneuron marker, and *Calb1* and *Pcp4* are markers for pyramidal cells in superficial and deep cortical layers¹¹, respectively.



markers known to co-exist with CCK in interneurons^{5,6}. None of the cells (0/45) contained parvalbumin (*Pvalb*) mRNA transcripts. By contrast, Exc-type pyramidal cells only expressed *Slc17a7*, and to a lesser extent *Slc17a6*, but not *Slc32a1* (0 of 38 cells), along with considerably lower copy numbers of *Cck* mRNA (Fig. 3e). Thus, our RNA-seq data accurately reflect the major functional distinction between inhibitory and excitatory neurons in the cerebral cortex. Examining the RNA phenotypes of each of the 5 I-types among interneurons revealed a distinct pattern of common molecular markers (Figs. 1g and 3e). Thus, at this level of analysis, there was a one-to-one correspondence between transcriptionally and electrophysiologically defined cell identities.

Mapping neuronal identities on single-cell RNA-seq data sets

Electrophysiology is inherently limited in throughput. Consequently, the molecular classification of neurons from small and/or heterogeneous groups of cells is challenging because of the resulting low statistical power. To increase the reliability of our molecular classification, we took advantage of the single-cell data set on somatosensory cortex we recently generated¹¹, containing >3,000 single-cell transcriptomes. We reasoned that if Patch-seq data could be aligned to this much larger data set, we would be able to assign electrophysiological properties to molecularly better-defined neuronal subclasses. Even though this approach is not mandatory for neuronal classification, the increasing availability of reference data sets for major brain regions will enhance overall classification accuracy in small-sized sample populations.

We built a correlation-based classifier to assign each neuron from Patch-seq to one of the possible neuronal subtypes distinguished earlier¹¹. The classifier used an iterative process of selecting relevant features (e.g., genes), ranking the candidate groups by correlation with any measured cell, and removing groups with lower correlation (Fig. 4a). None of the excitatory cells (0/38) were classified as interneurons, whereas a single interneuron (1/45) was found reminiscent of L5 pyramidal cells (Fig. 4b).

The anatomical position, electrophysiology classification and molecular phenotype of pyramidal cells with high certainty corroborated

their layer-specific L2/3-L5 identities (Fig. 4c). All of the measured interneurons were assigned to CCK⁺ subclasses (Fig. 4d). None of the “I-type” interneuron subdivisions was classified into a single group molecularly. Instead, I-types 1 and 5 were aligned to one set of closely related interneurons (Int11-Int14 in ref. 11; Fig. 4d). Likewise, I-types 2 and 3 were assigned to Int5-Int8, forming another subset of closely related cell types. I-type 4 showed exceptional heterogeneity with half of the cells assigned to each of these subsets. This shows that the combination of patch-clamp and RNA-seq methods benefits from an increased power of classification through the combination of real-life biophysical (and morphological) criteria and statistical predictions.

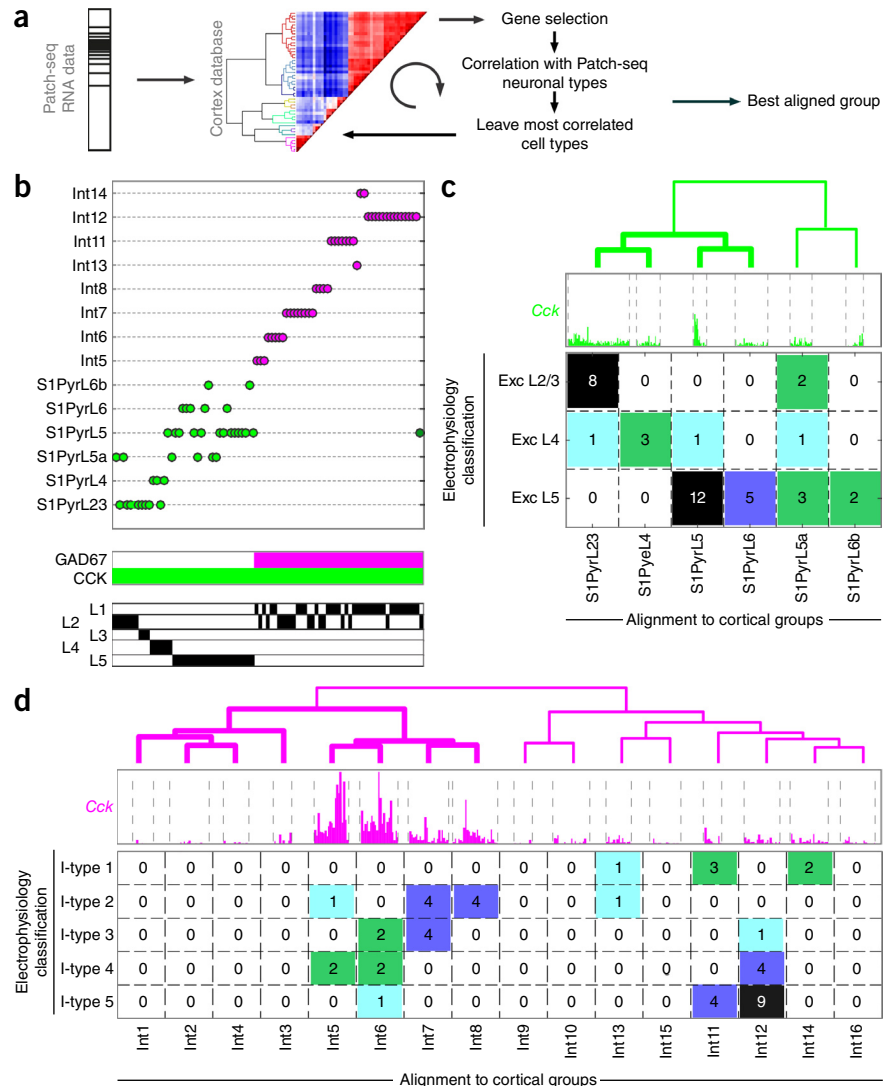
Molecular candidates to determine interneuron excitability

The depth of our molecular analysis allowed us to quantitatively assay the expression of channels, ion pumps and receptors in the I-type interneurons (Fig. 5a–c). Having our cells patch-clamp recorded, we could compare expression differences of any subunit detected with membrane potential changes (Fig. 5d). For example, Na⁺/K⁺ ATPase is a key electrogenic determinant of V_{rest} in excitable cells^{34,35}. The quantitative expression of genes encoding ATPase subunits (*Atp1a1-Atp1b3*; Fig. 5a) closely and positively correlated with the V_{rest} of each neuronal subtype in our data set (for *Atp1a3*, see Supplementary Fig. 3a).

Voltage-gated Cl⁻ channels, a family of poorly understood ion channels³⁶, are thought to modify V_{rest} by gating ion fluxes. Here, the quantitative expression of *Clcn3* was shown to positively correlate with V_{rest} (Supplementary Fig. 3b), potentially implicating these channels in determining subthreshold membrane potential fluctuations.

For AP frequency modulation, Kv3.1 (*Kcnc1*), a delayed rectifier K⁺-channel, is broadly assumed to confer the capacity to discharge at high frequencies upon prolonged depolarization of fast-spiking

Figure 4 Molecular classification and validation of Patch-seq-sampled neurons using a large cortical data set. (a) Schema of the correlation-based classification process used to align identified neurons in **Figure 1b** to previously described pyramidal cell (**Supplementary Fig. 1b**) and interneuron subtypes. (b) Classification results for each cell in our data set after fitting the best alignment. (c,d) Compliance of the electrophysiology-based classification with molecular clustering of pyramidal cells (c) and interneurons (d). Dendrograms depict cluster distances between neuronal subtypes (top). Bar plots denote *Cck* mRNA copy numbers in respective cell types (below).



interneurons³⁷. Our data support this because interneurons firing in excess of pyramidal cells expressed $\sim 3\times$ higher RNA count/cell for *Kcnc1* ($P < 0.05$ for the whole I-type cell population). Similarly, coherence of the electrophysiology and RNA data was demonstrated by detecting cyclic nucleotide-regulated ion channel *Hcn1*. We saw no expression in I-type 1 cells, where electrophysiology confirmed the lack of hyperpolarization-activated sag depolarization, a biophysical consequence of *Hcn1* activity (**Fig. 1b**; **Supplementary Table 1**). Thus, our RNA-seq data will allow predictions for future neurophysiology studies interrogating specific parameters of neuronal excitability.

Correlation matrix for use-dependent markers of neurons

Independent of many a priori classification, Patch-seq also permitted the analysis of correlations between gene expression and electrophysiological parameters. The quantitative expression of many genes (748 out of 5,600) showed significant correlation with one or more electrophysiology parameters. We took advantage of our quantitative data sets, and asked if rendering mRNA copy numbers of ion channels and synapse-related proteins (167 passed our criteria) as predictors returns meaningful association with specific biophysical parameters of single APs or AP trains. We hypothesized that any sufficiently robust correlation (filtered for correlation coefficients exceeding -0.4 and/or $+0.4$ (ref. 11); **Supplementary Fig. 4a,b**) could be valuable for future studies if it allows for distinguishing any CCK⁺ interneuron subtype. The quantitative expression of 24 genes (*Clic4*, *Clip3*, *Cacna1g*, *Kcnma1*, *Kcnj11*, *Kcnc1*, *Apba2*, *Cacna1g*, *Cadps2*, *Exoc8*, *Gria1*, *Grin2b*, *Htr7*, *Kcnma1*, *Npy*, *Pak1*, *Pcdh8*, *Slc32a1*, *Slc6a17*, *Sort1*, *Stx4a*, *Syt6*, *Syt7*, *Tac2*) had significant correlation with at least one electrophysiology parameter (**Supplementary Fig. 4d–i**). The main advantage of this approach is that it provides testable hypotheses by focusing on the preferential expression of genes in one but not another subset of interneurons. For example, synuclein- γ (*Sncg*), synaptotagmin 7 (*Syt7*), vesicle-associated membrane protein 4 (*Vamp4*) and the GABA synaptic reuptake transporter *Slc6a1* were grouped together and associated with AP parameters. Although the exact functional importance of these associations remains elusive, they provide candidates for the future molecular dissection of neuronal networks under physiological or pathological states.

Subtype-specific receptor repertoire in CCK interneurons

At any point in time, the intrinsic excitability of a neuron is dynamically tuned by its afferent inputs. An advantage of our Patch-seq data set is that it contains information on most (if not all) ligand-gated ionotropic channels, metabotropic (G protein-coupled) and other receptors, which determine the net network load on each sampled neuron (**Fig. 5a–c**). This allows for inferences to be made on the specificity and heterogeneity of afferent inputs.

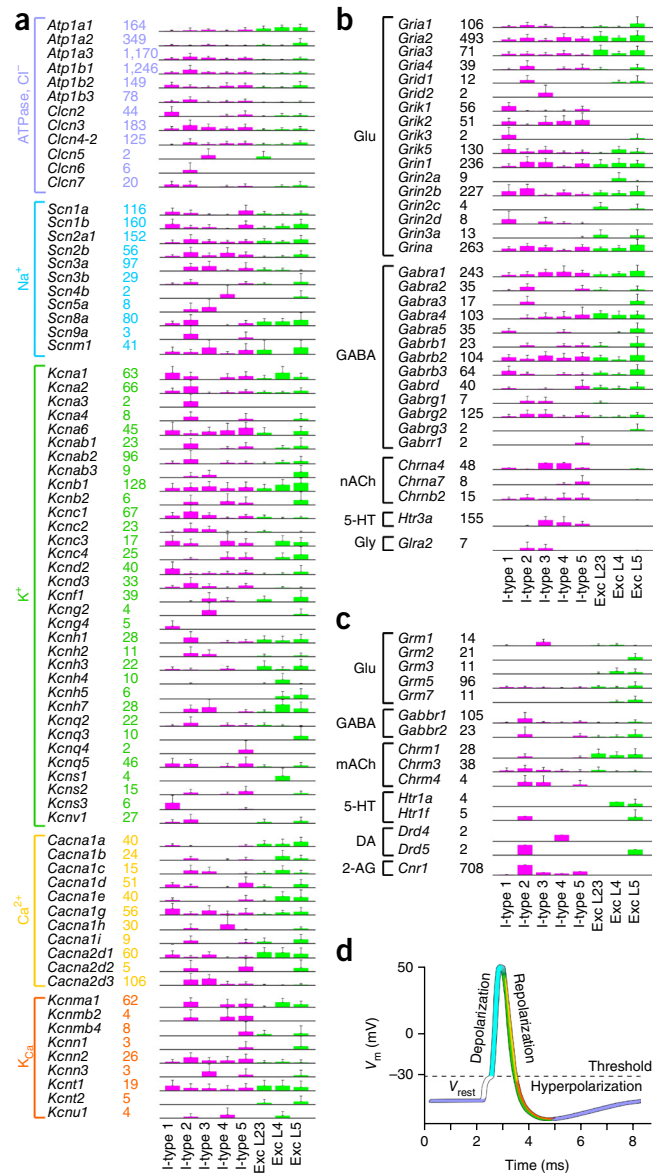
For example, fast glutamatergic transmission relies on α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors expressed by all neurons. Its subunits, GluR1 (*Gria1*)–GluR4 (*Gria4*), are differentially expressed in various neuronal subtypes. As such, the fast kinetics of AMPA receptor inactivation and desensitization in interneurons is usually explained by their low levels of GluR2 (*Gria2*) subunit³⁸. Here, we sampled 210 *Gria1* mRNAs in total in 45 interneurons (4.46 ± 5.3 mRNA molecules/cell) and 283 mRNAs cumulatively in 38 pyramidal cells (8.57 ± 8.08 mRNA molecules/cell; $P < 0.05$, mean difference of 3.61; **Fig. 5b**), thus quantitatively recapitulating earlier predictions by histochemistry and channel neurophysiology. Next, we examined the expression of the type 1 cannabinoid receptor (*Cnr1*) in our sample, which was reported to be highest in CCK⁺ interneurons in the brain^{39,40}. Here, we find that I-type 1 cells lack appreciable *Cnr1* mRNA expression (12 *Cnr1* mRNA molecules in total),

Figure 5 Cell type-specific quantitative expression of ion channel and receptor genes in CCK⁺ interneurons and pyramidal cells. **(a)** Differential expression of Na⁺/K⁺-ATPase, voltage-gated Cl⁻, Na⁺, K⁺, Ca²⁺ and Ca²⁺-activated K⁺ (K_{Ca}) ion channel subunits. **(b)** Ligand-gated ionotropic channel subunits of glutamatergic (Glu), GABAergic (GABA), nicotinic acetylcholinergic (nACh), serotonergic (5-HT) and glycinergic (Gly) receptors. **(c)** Subunits of Glu, GABA, muscarinic acetylcholine (mACh), dopamine (DA) and 2-arachidonoylglycerol (2-AG)-activated metabotropic receptors. Numbers correspond to mRNA molecule counts. Error bars, mean \pm s.d. Interneuron and pyramidal cell subtypes are color coded in magenta and green, respectively. **(d)** Schema of an AP indicating its specific phases: rest (V_{rest})/subthreshold (magenta), depolarization (cyan), repolarization (green/yellow) and hyperpolarization (green/orange). Color codes accord with those of the voltage-gated ion channels determining fast changes in membrane voltage.

which contrasts with I-type 2–5 subclasses (678 *Cnr1* mRNAs in total; **Fig. 5c**). These differences suggest another hitherto undescribed level of molecular complexity among cortical CCK⁺ interneurons.

Many developmental biology studies utilize the serotonin (5-HT) receptor 3a (*Htr3a*) subtype to detect and classify CCK interneurons^{4,41}. In our Patch-seq data set, we observed no 5-HT3a expression in I-type 1 cells (**Fig. 6a,c**), low *Htr3a* mRNA expression in I-type 2 (*Calb⁺/Npy⁺/Vip⁻*) cells and high mRNA copy numbers in I-type 3 (*Vip⁺/Npy⁻*) and I-type 4 (*Vip⁺/Npy⁺*, **Fig. 6b,c**) and I-type 5 (*Vip⁻/Npy⁺*) interneurons. This suggests that *Htr3a* expression among CCK⁺ interneurons might be more restricted than initially thought, likely leading to the undersampling of CCK interneurons by *Htr3a*-driven genetic tags⁴¹.

Subsequently, we expanded our analysis to all *Htr1a*–*Htr7* subtypes (**Fig. 6c**). None of the CCK⁺ interneurons contained *Htr1a*, *Htr1b* or *Htr1d* mRNAs. Notably, I-type 1 cells contained no mRNA copy for any of the 5-HT receptors. We validated our Patch-seq results by measuring the excitatory effect of 5-HT on I-type 1 ($n = 4$) versus I-type 4 ($n = 4$) interneurons (**Fig. 6a,b**) in whole-cell current-clamp experiments. While clamping the cells to 0 pA, 5 μ M (data not shown) and 25 μ M 5-HT (**Fig. 6d,e** upper trace) were bath-applied. Both subthreshold depolarization with 5 μ M and suprathreshold depolarization with 25 μ M 5-HT^{42–44} was observed in both I-type 1 and 4 interneurons. To dissect the origin of the depolarizing 5-HT effect and rule out indirect effects imposed by the neuronal network into which the recorded cell was embedded, 10 μ M 5-HT was microejected ('puffed') onto interneuron somata. We controlled equal 5-HT load by coapplying a fluorescent tracer (**Fig. 6d,e**, middle). Direct 5-HT application onto I-type 1 interneurons did not cause depolarization (**Fig. 6d**, bottom). By contrast, I-type 4 interneurons became readily depolarized (**Fig. 6e**, bottom). Our pharmacological results thus establish causality, considering that RNA-seq predicts 5-HT insensitivity in I-type 1 interneurons owing to the lack of any metabotropic or ionotropic 5-HT receptors. Moreover, the morphological reconstruction of the sampled interneurons (**Fig. 6a,b**) is explanatory of the depolarization of I-type 1 cells upon bath application of 5-HT. I-type 1 interneurons were dye-coupled to neighboring cortical nonpyramidal neurons, and even pyramidal cells. This allowed for interneurons to synchronize their membrane potentials through gap junctions⁴⁵, assigning I-type 1 cells as passive followers of network depolarization at suprathreshold 5-HT load. Thus, our Patch-seq data primed us to identify a CCK⁺ interneuron with a 5-HT-driven effector behavior in its L1 microcircuit even if the interneuron itself lacked 5-HT receptors. Overall, we suggest that our Patch-seq approach will help future systems neurobiology investigations to rationalize diverse functional outcomes by providing a platform of identifying marks for specific modalities among neurons.

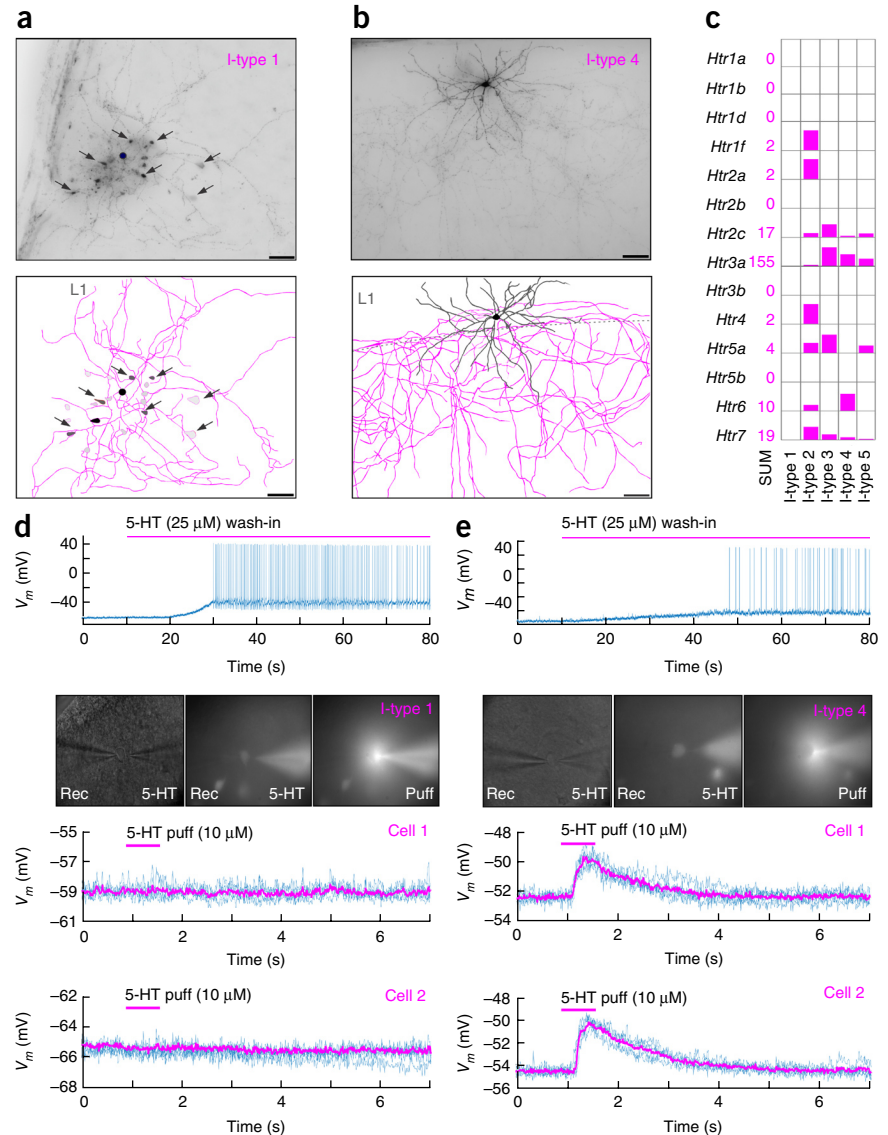


DISCUSSION

The brain undoubtedly exhibits the highest level of cellular heterogeneity¹⁰, and contains a large variety of neurons that differ in their morphology, connectivity, biophysical parameters and molecular phenotypes^{5,6,46}. The taxonomy for neurons dates back to the first pioneers of neuroanatomy (e.g., Cajal and Golgi), who exclusively used morphological features, such as the size and topography of axonal and dendritic arbors, for classification and is now based on a wide array of neurophysiology, advanced histochemistry and RNA analyses⁶. Nevertheless, reliance on known candidate marks continued to dominate, and delayed the inception of unbiased classification. Moreover, the limited number of markers that could be probed at any given time (~ 20 for single-cell PCR and histochemistry)⁷ together with the often mutually exclusive experimental conditions that neurophysiology and single-cell molecular biology tools require limited detailed fingerprinting of cellular components in the brain.

We combined mouse genetics and patch-clamp electrophysiology to successfully target a distinct cohort of interneurons^{5,47} to overcome existing limitations of classical function-structure analyses.

Figure 6 Compliance of RNA-seq predictions with the neurophysiological phenotype of cortical interneurons. **(a)** Orthogonal image stack of DAB-stained I-type 1 interneuron (top) and reconstruction of its processes, including dye-coupled neighbors (arrows, bottom). Scale bars, 100 μm . **(b)** I-type 4 interneuron processed as above (top) and its reconstruction (bottom). Scale bars, 100 μm . **(c)** Subclass-specific quantitative assessment of mRNA expression for ionotropic and metabotropic 5-HT receptor subunits in interneurons. **(d,e)** Top: representative AP firing by I-type 1 **(d)** and I-type 4 **(e)** interneurons upon bath-applied 5-HT (25 μM). Middle: differential interference contrast and epifluorescence microscopy images (60 \times) of the recorded cells, suggesting identical 5-HT (10 μM) load upon focal microejection (puff). Bottom: membrane potential responses to focal 5-HT application. Two examples (cell 1,2) per group are shown. Rec, recording plane.



In the CCK⁺ cell population studied, we expected considerable neuronal diversity, which allowed us to improve and optimize Patch-seq to its present accuracy, even when low copy numbers of mRNA molecules were present. We first classified our CCK⁺ interneuron sample from L1/L2 (but not from deep cortical layers¹¹ or hippocampal subfields^{5,47}) into the five commonly identified subtypes. We further showed that Patch-seq can identify a select number of molecular determinants that can be used to further subdivide CCK⁺ interneuron subclasses. Each of these examples is important because they reconcile previously reported sets of data on receptors (*5Htr3a*, *Cnr1*) and channels (*Hcn1*) (refs. 3,6,7,39–41). We note that Patch-seq can discover sets of cellular markers independent of other classification systems or a priori knowledge of the cell type of interest. We also show that Patch-seq can help to form hypotheses, for example, about neurotransmitter-receptor relationships. We expect that similar approaches can be applied to essentially any neuronal subtype, and will help to prevent false-negative results (due to undersampling of neuronal contingents) in cases when the resolution of available histochemical tools is limited or if suitable reagents are not available. The lack of *Rgs12*, *Htr3a* and *Cnr1* mRNA transcripts in I-type 1 interneurons raises the possibility that this abundant subclass might have been systematically missed in prior genetic reporter analyses, obscuring their contribution to fundamental cortical network events.

The efficiency of mRNA capture in Patch-seq is lower than that in single-cell RNA-seq on dissociated tissues. However, it is still sufficient to efficiently sample even genes of low expression because of its extremely low rate of false-positive identification^{11,12}. Thus, mRNA copy numbers even at the range of 1–5 molecules return meaningful associations. Moreover, the combination of Patch-seq with transgenic mouse technologies might allow the future exploitation of external (spike-in) reference standards (e.g., transgene products), thus facilitating positive cell identification. These methods, together with the progressive decoding of regional heterogeneity in the nervous system through large-scale RNA-seq databases^{11–13}, can increase the

stringency of neuronal classification. Such reference atlases, once available, will allow for precise hierarchical landscapes to be built even when cell numbers from patch-clamp electrophysiology experiments are limited. However, Patch-seq can stand alone and give much more complete and accurate information about gene expression (~2,000 genes per cell) in selectively probed cell contingents, compared to previous methods (e.g., qPCR for 10–20 genes/cell)^{6,7}.

Patch-seq samples somatic material upon aspiration. In neurons, dendrites and axons occupy large spaces and their intracellular volume is considerable. Therefore, one may argue that Patch-seq misses many mRNAs that are preferentially targeted to distant domains of axons or dendrites. Although some mRNA is actively transported into neurites⁴⁸, this does not mean that those mRNA species are absent from the soma. On the contrary, most (if not all) mRNA species are more abundant in the soma than in neurites, and there is not a single known case of an mRNA that is localized exclusively outside the soma. This is true even for mRNAs thought to be actively transported into neurites, such as *CamKII α* and *spinophilin*⁴⁹. It is also known that the axon and dendrite contain much less total mRNA than the soma: axons are thought to contain about 1,000–4,500 mRNAs, and dendrites contain >2,500 mRNAs⁴⁹. These numbers are at least an order

of magnitude lower than that of the soma (which contains >100,000 mRNAs). As a result, sequencing the soma content can be expected to give a representative view of mRNA expressed by a neuron, although without information on which mRNAs are more or less efficiently transported into the neurites.

Another technical element that needs to be tightly controlled is the length of electrophysiology recordings because electrical stimuli might alter the transcriptome. Here, we used 20- to 25-min protocols, as the lifetimes of most mRNA molecules are on the order of many hours (median: 9 h), with none known to be shorter than 1 h⁵⁰. The quickest transcriptional response known in any setting is the induction of immediate-early genes (e.g., *c-Fos* (also known as *Fos*), *Jun*, *Egr2*), which can be detectable after 30 min, but peaks at 3 h. However, removing immediate-early genes did not affect cell-type identification. Thus, the impact of patch-clamp recordings that occur on a timescale shorter than 1 h can be expected to have minimal impact on the RNA transcriptome.

In conclusion, Patch-seq can be expected to facilitate the characterization of transcriptome-wide changes in many experimental settings, thus contributing to a better understanding of fundamental physiological and pathological processes.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Accession codes. GEO: [GSE708744](#).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.F., A.Z., S.L. and T.H. wrote the paper. J.F. performed electrophysiology and the electrophysiology-based cell classification, and drafted figures. A.Z. performed single-cell RNA-seq and the transcriptome-based cell classification, and drafted figures. D.C. performed *post hoc* morphological reconstruction of biocytin-filled neurons. Y.Y., Z.M. and G.S. provided unique reagents. S.L. and T.H. acquired funding and oversaw the research. All authors read and approved the manuscript for submission.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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following the lysis step, 2 μ l RT mix (1 \times SuperScript II First-Strand Buffer; Life Technologies) supplemented with 10.6 mM MgCl₂, 3.6 μ M template-switching oligo C1-P1-RNA-TSO 5'-Bio-AAUGAUACGGCGACCACCGAUNNNNN NGGG-3', 1.5 U/ μ l TaKaRa RNase inhibitor (Clontech), 1.45 M betaine and 21 U/ μ l Superscript II reverse transcriptase (Life Technologies) were added and incubated at 42 °C for 90 min followed by 72 °C for 10 min. Following reverse transcription, 8 μ l PCR mix (1 \times KAPA HiFi 2 \times ready mix and 240 nM C1-P1-PCR2 5'-Bio-GAATGATACGGCGACCACCGAT-3') were added and PCR-amplified using thermal cycling as follows: 95 °C for 3 min (5 cycles), 98 °C 20 s, 62 °C 4 min, 72 °C 6 min, (9 cycles) 98 °C 20 s, 68 °C 30 s, 72 °C 6 min, (7 cycles) 98 °C, 30 s, 68 °C 30 s, 72 °C 7 min. Subsequently, PCR samples were cleaned using AMPure-XP beads (1:1 ratio; Beckman Coulter) and quantified by qubit (Life Technologies) on an Agilent bioanalyzer. Library preparation was done using tagmentation as described²⁹.

Illumina sequencing. Libraries were sequenced on an Illumina HiSeq2000 instrument using C1-P1-PCR2 as read1 (50 nt) primer and C1-TN5-U PHO-CTGTCTCTTATACACATCTGACGC as index read (8 nt) primer.

Data analysis. Read processing and molecule counts were performed as reported recently¹¹. We only analyzed cells with >1,500 mRNA molecules/cell (excluding mitochondrial, repeat and rRNA) and if a complete catalog of patch-clamp read-outs was available.

Alignment of interneurons and pyramidal cells on a cortical template. We used our recently described cortical data set¹¹ to resolve each of the interneurons and pyramidal cells into one of the template groups (from >3,000 dissociated cells). First, we narrowed down our search to one of the layer-specific pyramidal cell cohorts (S1PyrL1-L6) or interneuron groups (Int1-16), 22 groups in total. Because of a significant difference in the number of mRNA molecules detected, we designed our classifier on correlation measures rather than Euclidian distance. As feature selection is an important parameter for classification

(e.g., which genes are “in use”), the classifier continuously updated the features for the groups compared. First, the median expression for every group (in the cortex data set) was calculated. Because the s.d. of the genes' median expression (along groups) did not depend strongly on their mean expression, we selected genes (features) using a fixed threshold of s.d. being >1.5 over the groups compared. The process was as follows: (i) selection of a Patch-seq cell for classification; (ii) setting the list of candidate groups to contain all 47 neuronal groups of the somatosensory cortex; (iii) selection of genes for the current iteration, $\text{std}_{(\text{gene median expression})} > 1.5$; (iv) calculation of the correlation between a Patch-seq cell and all candidate groups; (v) ranking neuronal groups by their correlation to the Patch-seq cell (highest to lowest); (vi) removal of those candidate groups that were <50% with their correlation; (vii) if the list of candidate groups contained more than one group we looped back to step iii. If only a single candidate group was left, the Patch-seq cell was assigned to that particular group.

Correlation of gene expression and electrophysiology parameters. This analysis was aimed to identify mRNAs that might be predictors for electrophysiology parameters. We focused on mRNAs coding for ion channels, receptors and synaptic transmission-related proteins as sets of genes whose biological interpretation might be tested experimentally. We tested the correlation between all genes (5,600) that passed our baseline criteria (that is, more than five cells with nonzero expression, ~12% of data points) against all electrophysiology parameters (110). Along with the correlation, *P*-values for the null hypothesis of independent variables were calculated (permutation *P*-value showed similar results). For each gene separately, we used a false recovery rate (FDR) of 10% to declare a significant correlation. This was performed separately for every gene because otherwise the *P*-values strongly correlated and the assumptions for FDR were violated. Next, we focused only on ion channels, receptors and synapse-related genes (167 of these passed our above criteria). Correlation coefficients shown in **Supplementary Figure 4** are for genes that exhibited correlation (or anti-correlation) greater than 0.4.