Landscape of ribosome-engaged transcript isoforms reveals extensive neuronal-cell-class-specific alternative splicing programs

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Nervous system function relies on complex assemblies of distinct neuronal cell types that have unique anatomical and functional properties instructed by molecular programs. Alternative splicing is a key mechanism for the expansion of molecular repertoires, and protein splice isoforms shape neuronal cell surface recognition and function. However, the logic of how alternative splicing programs are arrayed across neuronal cell types is poorly understood. We systematically mapped ribosome-associated transcript isoforms in genetically defined neuron types of the mouse forebrain. Our dataset provides an extensive resource of transcript diversity across major neuron classes. We find that neuronal transcript isoform profiles reliably distinguish even closely related classes of pyramidal cells and inhibitory interneurons in the mouse hippocampus and neocortex. These highly specific alternative splicing programs selectively control synaptic proteins and intrinsic neuronal properties. Thus, transcript diversification via alternative splicing is a central mechanism for the functional specification of neuronal cell types and circuits.

The mammalian brain contains hundreds of cell types with unique anatomical and functional properties. Cell type characteristics are fundamental underpinnings of neuronal circuit function and, ultimately, the control of behaviors. Many of the distinctive neuronal morphologies were recognized 100 years ago. More recent studies have uncovered electrophysiological properties and characteristic gene expression profiles that are associated with specific neuron types. Yet, we still lack comprehensive knowledge of how the multitude of neuronal properties is encoded by a limited number of genes. Evolutionary comparisons have revealed a significant increase in alternative splicing heterogeneity in more complex organisms. Within those, the nervous system exhibits the most extensive usage of alternative transcript isoforms. Single-gene studies have provided evidence that individual protein variants generated through alternative splicing can exhibit unique isoform-specific functions. However, in many molecular studies of neuronal connectivity and function, the identities of the splice isoforms that are endogenous to the cell type of interest are unknown. This is a significant bottleneck for interpretation of gain-of-function and loss-of-function studies. While loss of RNA-binding proteins (RBPs) that regulate alternative splicing and cell-type-specific knockouts exhibit severe impacts on neuronal function and synaptic transmission, most of these proteins are commonly expressed in all neuronal cell types. Thus, the general logic of how alternative splicing programs relate to brain complexity is poorly understood.

Previous bulk-sequencing analyses contrasted neuronal and non-neuronal splicing regulation. Developmental analyses of the mouse neocortex uncovered a series of temporally controlled coordinated splicing switches in brain tissues. These developmental switches were pan-neuronal and occurred across all neuronal populations. Only very recently have studies begun to probe whether well-defined neuronal cell types rely on alternative splicing for the regulation of specific biological functions. However, it remains debated as to what extent transcript isoforms detected by RNA-sequencing (RNA-seq) are indeed recruited for translation to produce protein isoforms. To address these questions, we generated genome-wide maps of transcript isoforms that are recruited for translation in genetically defined neuronal cell populations. Our analysis identified hundreds of differentially regulated (DR) splicing events across distinct neuron types. Moreover, we demonstrate that cell-type-specific splice isoforms define neuronal cell populations and shape intrinsic properties and synaptic protein complexes. This dataset provides a rich resource for selecting endogenously expressed splice isoforms to be used in functional studies, for interpreting the impact of gene mutations on disease states and for the dissection of enhancers and promoters that drive cell-type-specific transcripts from alternative transcription start sites (TSSs).

Results
Deep mapping of actively translated transcript isoforms in cortical and hippocampal neuron populations. To obtain a comprehensive map of transcript isoforms in the mouse forebrain, we conducted large-scale tagged-ribosomal affinity purification (RiboTRAP) of ribosome-associated mRNAs from genetically defined neurons (Fig. 1a). The endogenous ribosomal protein Rpl22 was conditionally tagged with hemagglutinin (HA) in glutamatergic neurons (using calcium/calmodulin-dependent protein kinase II (CaMKII)-cre for most neocortical pyramidal cells and sodium channel, nonvoltage-gated 1α (Scnna1a)-cre for spiny stellate and star pyramid layer 4 (L4) cells) and in GABAergic interneurons (with somatostatin (Sst)-cre, parvalbumin (Pvalb)-cre and vasointestinal peptide (Vip)-cre). Within the hippocampus, we further targeted cornu Ammonis 1 (CA1) neurons (CaMKII-cre), CA3 neurons (glutamate receptor, ionotropic, kainate 4 (Grik4)-cre) and Sst-positive interneurons (Sst-cre) (Fig. 1b; Supplementary Fig. 1). Using an optimized affinity-isolation protocol and strict quality control measures followed by deep RNA-seq (paired-end, read length of 100 base pairs, >100 million reads per biological replicate), we detected >12,000 genes per sample with full-length coverage across transcripts (Supplementary Figs. 2 and 3a) and low variance.
between biological replicates (Fig. 1c). The transcriptome analysis confirmed appropriate enrichment and de-enrichment of known and newly discovered markers. Widely expressed non-neuronal genes such as astrocyte markers were either not detected or showed low-level background in some of these isolates (Supplementary Figs. 1b, 3b–d and 4; Supplementary Table 1). Thus, this deep dataset enables a reliable dissection of transcript isoforms that are translated in specific cell types.

**Alternative transcript repertoires define neuronal populations.** To map transcript repertoires across neuronal cell types, we quantified alternative isoforms using two complementary computational methods. First, we analyzed differential exon usage by quantifying reads mapping onto individual exons relative to the number of reads on constitutive exons derived from the same gene (constitutive exons in the caudal ganglionic eminence (CGE)-derived Vip interneurons were distinct from Scnn1a-defined interneuron classes (Pvalb and Sst populations) at significance level of 10−1, P ≤ 0.01). Independent experimental validations using semi-quantitative PCR with reverse transcription (RT–PCR) confirmed the accuracy of the computational pipeline (validation rate >90%; Supplementary Fig. 6). Therefore, this validated dataset represents a comprehensive resource for alternative transcripts in neuron populations of the major forebrain (Supplementary Tables 2 and 3; see also https://scheiffele-splice.scicore.unibas.ch for a web-based look-up tool to query isoforms for individual genes). Divergent transcript isoforms may arise from both alternative splicing and alternative TSSs. The Pattern analysis enabled us to separate transcript isoforms arising from these mechanistically different forms of transcript diversification. Remarkably, the exons DR by alternative splicing reliably segregated neuronal cell classes (Fig. 1d). Thus, Scnn1a-defined L4 cells are characterized by 310 exons included in 214 different isoforms in neocortical and hippocampal cell populations (log, fold-change (FC) in the splicing index (SI) of ≥1 or ≤−1, P ≤ 0.01).
in 407 genes. Overall, we did not observe a correlation of changes in splicing indices and gene expression level, which indicated that our analysis captures DR exons across a broad spectrum of transcript expression levels (Supplementary Fig. 7). In previous studies, microexons (defined as exons that are 3–27 nucleotides long) were shown to preferentially contribute to transcript diversification in the nervous system. Among all exons that were DR across neuronal cell classes, we found that 3.8–5.3% were microexons. These percentages are slightly higher compared with the percentage of total microexons detected in the neocortex (2.8%; see Methods for details). Thus, differential alternative splicing across cell types is not only substantial for microexons but also for other types of splicing events. In summary, this analysis demonstrates that extensive alternative splicing across cell populations (Fig. 2a,b, see Dlgap2 as an example). Interestingly, alternative last exons (ALEs), which can result in a modification of the 3′ untranslated regions (3′ UTR) of transcripts, represented ~20% of events (Fig. 2a,b, see Ncam1 as an example). This is notable considering that ALEs can impose cell-type-specific protein expression as well as subcellular localization of mRNAs. In addition to transcript diversification via alternative splicing, we found that across all neocortical cell classes, ~30–60% of DR transcript isoforms arose from alternative TSSs (Fig. 2c; Supplementary Table 2). This result implies that there is frequent action of cell-type-specific enhancers and promoters. An example for alternative TSS regulation is Dlgap1, which encodes a major glutamatergic scaffolding protein. In neocortical Pvalb-positive cells, we identified an alternative TSS in exon 3 of Dlgap1, which switches to exon 5 in the Scnn1a-positive L4 cells (Fig. 2d). This differential regulation results in transcripts that differ in the 5′ UTR and the amino-terminal amino acids (Fig. 2d, see Rapgef5 for an additional

Wide use of alternative TSSs across cortical neuron subclasses. We quantified the frequency of distinct patterns underlying the DR splicing events in neocortical cell populations and found that they distributed over multiple categories (Fig. 2a; Supplementary Table 2). Usage of cassette exons was the most frequent DR alternative splicing event across cell populations (Fig. 2a,b, see Dlgap2 as an example).
Divergent alternative splicing programs across closely related cells in different anatomical positions. Hippocampal CA1 and CA3 pyramidal neurons exhibit certain unique functional properties and overall similar transcriptomes. Thus, we explored whether there are differential splicing programs that are specific for these closely related glutamatergic cell classes. We identified hundreds of differentially expressed transcript isoforms arising from different patterns of alternative splicing, as well as alternative TSSs between CA1 and CA3 cell preparations (253 DR exons, log2(FC) ≥ 1 and ≤ -1, P ≤ 0.01; Fig. 3a, Supplementary Fig. 9, Supplementary Table 3). These include key isoforms with well-characterized functional properties, such as the mutually exclusive exons that regulate flip/flop variants of the Gria1 AMPAR subunit or ALEs in BCAN, which control the expression of a secreted isoform of the protein Brevican implicated in neuronal adhesion (Fig. 3b). Similarly, cortical L4 excitatory neurons and hippocampal CA1 pyramidal neurons exhibited 276 DR exons (Fig. 3a) and transcript isoforms derived from multiple patterns of alternative splicing (Fig. 3c, with Nrnx3 as an example; see also Supplementary Fig. 9 and Supplementary Table 4). These comparisons highlight the vastly divergent transcript isoform content between different classes of glutamatergic neurons, including closely related pyramidal cells from hippocampal subfields. By comparison, splicing programs were much more similar examples). When comparing the segregation of differential TSSs and ALEs, we found that either of these types of events efficiently segregated neocortical excitatory and inhibitory cell classes, including MGE- and CGE-derived interneurons (Supplementary Fig. 8a and b, respectively; the exon numbers involved are indicated in the figure legend). Thus, our analysis demonstrates that both alternative splicing and alternative TSSs are major drivers of neuronal-cell-type-specific transcript isoform expression in the mouse forebrain.
between hippocampal versus neocortical Sst-positive interneurons (only 151 highly DR exons; see Fig. 3a,c for cassette exon regulation in Fat1 as an example, and Supplementary Fig. 9 and Supplementary Tables 4 and 5 for an overview of differentially expressed genes and alternatively regulated splicing events). In summary, we conclude that alternative splicing plays a major role in diversifying molecular repertoires at the level of neuronal subclasses and cell types within and across anatomical positions.

Identification of neuronal-subclass-specific splicing factors in neocortical and hippocampal cells. Most neuronal RBPs studied thus far are pan-neuronally expressed. Given the extensive differential alternative transcript regulation in neuronal subclasses, we sought to identify RBPs that might regulate alternative splicing in a cell-type-specific manner. We generated a hand-curated list of 57 bona fide splicing regulators based on databases and previous publications and evaluated their expression across neuronal populations (Supplementary Table 6). As expected, several splicing factor transcripts exhibited broad expression, with little difference across neocortical and hippocampal cell classes (for example, Hnrrnap2b1, Hnrnpl and Srrm1; Fig. 4a, Supplementary Table 6). By contrast, other splicing factors showed highly selective expression, with some segregating between glutamatergic and GABAergic neuron groups and others highly enriched in certain neuron classes (Fig. 4a).

For example, the Rbm20 transcript is preferentially expressed in Pvalb interneurons, while Ptpb1 is preferentially expressed in Vip interneurons and Rbfox3 (also called NeuN) is preferentially expressed in glutamatergic cells (Fig. 4a; Supplementary Table 6). Fluorescence in situ hybridizations for select RBPs confirmed the differential expression patterns extracted from RiboTRAP sequencing data (Supplementary Fig. 10; see Supplementary Table 6 for statistical analyses). To investigate whether some of the differentially expressed splicing factors represent candidates that drive cell-type-specific alternative splicing choices, we employed splice reporter assays in neuroblastoma 2A (N2A) cells (see Methods for details). We generated reporter constructs for exons that we found to be differentially included across neocortical neurons (alternative cassette exons in the neurotransmitter receptor-encoding transcripts Gabrg2 and Grin1, and the voltage-gated potassium channel-encoding transcript Kcnq2; Fig. 4b). Co-expression in N2A cells of several RBPs (for example, hnRNP A1, hnRNP H1 and Ptbp3) did not shift splicing patterns in vitro. Conversely, co-expression of Ptbp1 (which is preferentially expressed in Vip interneurons; Fig. 4a,d) shifted the splicing of Gabrg2 and Kcnq2 reporters to the pattern observed for endogenous mRNAs in Vip interneurons (Fig. 4c). Similarly, Rbfox3 shifted the Grin1 reporter splicing to the pattern enriched in Scnn1a cells (which express high levels of Rbfox3) (Fig. 4c,d). Thus, these differentially expressed splicing regulators represent possible candidates for the modulation of the respective splicing events in vivo. In summary, this analysis identified candidate neuronal cell-class-specific splicing regulators for the differential regulation of transcripts.

Alternative splicing programs are highly dedicated to controlling synaptic interactions and neuronal architecture. To probe which cellular properties are regulated by alternative splicing, we assessed the enrichment of Gene Ontology (GO) terms for transcripts regulated across all neocortical cell classes, hippocampal comparisons and across brain regions. Given that alternative first exons result from transcriptional regulation, we excluded them from this analysis. Remarkably, the differential alternative splicing regulation almost exclusively targeted transcripts that encode synaptic proteins and intrinsic neuronal properties. Enrichment of the top GO terms significant for genes that are DR by splicing was 2–4-fold higher compared with genes that were differentially expressed (Fig. 5a; see Supplementary Fig. 11a,b for enriched categories of differentially expressed genes and Supplementary Table 7 for all GO terms). Note that the enrichment of genes that encode synaptic proteins was not simply a consequence of such genes containing larger numbers of exons (Supplementary Fig. 11c). Specifically, the enriched GO terms map onto the following five key categories, which fundamentally shape synapse function and intrinsic neuronal properties: adhesion complexes (for example, Ctn4, Ctnnap2, Naml1, Ngln1, Nrxn3, Nfasc, Ptprs and Robo2) implicated in the formation and specification of neuronal synapses; voltage-gated calcium channels (for example, Cacnb2, Cacnb4, Cacna1g and Cacna1d); presynaptic release machinery (for example, Rims, Synj1, Stxbp1, Syt17 and Unc13b); postsynaptic neurotransmitter receptor complexes (for example, Grm1, Grm5, Gria1, Gria2 and Shisa9); and associated scaffolding proteins (for example, Camk2, Dlgap1, Rapgef4, Shank3 and Tiam1) (Fig. 5b). All of these genes encode key regulators of synaptic function and plasticity. Three further categories that were highly targeted by cell-type-specific alternative splicing are potassium channels, motor proteins and regulators of cytoskeletal rearrangements, all of which are elements that are central for the control of intrinsic neuronal properties (Fig. 5c). In particular, potassium channels are key determinants of neuronal excitability at the level of after-hyperpolarization following action potential firing (Kcnm2), at the level of M-currents (Kcnq2) or through calcium-dependent...
regulation of A-currents (Kcnip1 and Kcnip4)\(^3\). Thus, neuronal-cell-type-specific alternative splicing programs specifically encode intrinsic neuronal properties and synapse specification.

**Discussion**

Previous studies have highlighted an expansion of splicing complexity across vertebrate species, with a particular increase in alternative
Fig. 5 | Alternative splicing programs are highly dedicated to the control of synaptic interactions and neuronal architecture. 

a. Heatmap representing the fold-enrichment of GO terms for transcripts regulated at the gene expression level or by alternative splicing identified by the Panther classification system (see Methods for details). Terms listed were selected based on the splicing analysis and had to be significant in at least one neocortical population, hippocampal comparison or comparison across brain regions. Corresponding values from analysis of differentially expressed genes are included on the left. Fields for the statistically significant enrichments (Fisher’s exact test with Benjamin–Hochberg FDR correction, P ≤ 0.05) are highlighted by bold outlines. Overall, transcripts undergoing differential alternative splicing show higher fold-enrichments compared to differentially expressed genes. Splicing-dependent transcript isoforms in Vip interneurons present lower fold-enrichment, whereas Scrn1a and Pvalb exhibit higher fold-enrichment of GO categories. See Supplementary Table 7 for the raw output from the GO analysis. 

b. Cartoon illustrating the main categories of genes whose alternative splicing is DR between cell populations (log2(FC) ≥ 1 and ≤ -1, P ≤ 0.01). Among the most enriched categories, we found genes encoding presynaptic proteins modulating calcium influx or vesicle fusion, presynaptic and postsynaptic adhesion molecules and postsynaptic scaffolding molecules. 

c. Cartoon illustrating examples of differentially expressed transcript isoforms encoding proteins that modulate intrinsic properties of neurons (for example, potassium channels, proteins involved in cytoskeletal remodeling and cellular transport along neurites).

exon usage in the brain. This increase in alternative splicing may relate to neuronal cell types and functions in multiple ways. Single-genome studies have illustrated stochastic splice isoform choices at the single-cell level, but also reproducible splicing patterns linked to cell types. Here, we demonstrated that complex alternative splicing programs define subclasses of cortical and hippocampal neuron types. Thus, the selection of cell-type-specific transcript variants is not an exceptional feature for individual protein families but a fundamental program of highly differentiated cell types in a complex organism. During embryonic development, the lineage decisions for interneuron and pyramidal cell differentiation are mainly driven by transcription factor codes. We propose that cell-type-specific expression of RBPs imposes splicing-dependent regulation for the terminal differentiation of these neuron classes. Consistent with this notion, several of the candidate splicing specificity factors that we mapped here are already detected in interneurons at embryonic stages of development.

The RiboTrap approach used in our study facilitates the interrogation of splice isoforms with excellent coverage across the entire transcript. Low-level background contamination for some cell classes may influence detected splicing differences, particularly for widely expressed non-neuronal genes. However, the RiboTrap approach benefits from deep coverage of splice junctions and rare transcript isoforms that go undetected or cannot reliably be quantified from single-cell sequencing data. Moreover, mapping ribosome-associated mRNAs focuses the analysis on transcript isoforms that are recruited by the translational machinery.

An unexpected finding in our study was the highly divergent usage of alternative TSSs across neuronal populations. This suggests prominent roles for cell-type-specific enhancers and promoters in generating transcript isoforms. We propose that this complex transcript regulation evolved not only to modify protein isoforms but also to afford unique spatiotemporal modulation of neuronal gene expression. Complex alternative splicing programs control diverse biological processes from chromatin and RNA regulators to ion homeostasis and mitochondrial function. Considering this broad range of splicing-regulated processes, it is remarkable that the neuronal-cell-type-specific splicing programs are selectively geared to the control of synaptic and intrinsic neuronal properties. Splice isoforms of neuronal receptors, ion channels, synaptic adhesion and scaffolding proteins exhibit fundamentally divergent functions and significant disruptions to splicing have been linked to neurodevelopmental disorders. In humans, more than 90% of gene products are modified by alternative splicing. A major impediment to exploring the functional relevance of transcript isoforms in neuronal wiring has been the lack of knowledge of how splice isoforms are arrayed over neuronal cell types. Our comprehensive genome-wide analysis uncovers hundreds of cell-class-specific transcript isoforms...
that encode key regulators of synaptic function and intrinsic neuronal properties. To maximize the accessibility of this large dataset and to simplify the identification of differentially expressed transcript isoforms, we have established a web-based SpliceCode database where users can retrieve differential isoform expression data for any gene of interest (https://scheiffele-splice.scicore.unibas.ch).

In the future, targeted manipulation of cell-type-specific splicing events may open the door for a new class of therapeutic interventions in disease states.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41593-019-0465-5.

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Author contributions
This work was jointly conceived by E.F., L.T. and P.S. All wet lab procedures were performed by E.F. and L.T., and data analyses were conducted by E.F., L.T. and G.F. Website design was performed by G.F., and the manuscript was jointly written by E.F., L.T. and P.S., with editing provided by G.F.

Competing interests
The authors declare no competing interests.

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Methods
Mice. All procedures involving animals were approved by and performed in accordance with the guidelines of the Kantonales Veterinäramt Basel-Stadt. Male and female mice were used in this study. RpL22-HA (RiboTag) mice, Povlb-cre mice, Sitt-cre mice, Camk2-c cre mice, Grk4-cre mice, Vip-cre mice and Sema7a-cre mice were obtained from Jackson Laboratories (jax stock numbers: 011029, 017320, 013044, 005359, 006474, 031628 and 009613, respectively). All lines were maintained on a C57BL/6J background. The specificity of cre-liners for recombination of the RpL22 allele was confirmed by immunohistochemistry and matched previous reports in the literature.

Immunohistochemistry and imaging. Animals (males and females) from postnatal days 25–42 were transcardially perfused with fixative (4% paraformaldehyde in 100 mM phosphate buffer, pH 7.4). The brains were post-fixed overnight in the same fixative at 4 °C. Coronal brain slices were cut between Bregma –1.43 and –2.15 (including the somatosensory cortex and the dorsal hippocampus) at 50 µm with a vibratome (Leica Microsystems VT1000). For immunohistochemistry, brain sections were kept in PBS before incubation for 1 h with blocking solution containing 0.05% Triton X-100, followed by incubation for 2 h at room temperature with a secondary antibody. Sections were washed three times in PBS before mounting onto microscope slides with Fluoromount-G (SouthernBiotech, 0100–01). The primary antibody used in this study was rat anti-HA (Roche, 11867430601; 1:100). The secondary antibody used was donkey antirabbit Alexa 488 (Jackson ImmunoResearch, 712–165–153; 1:100). Hoechst dye was co-applied with the primary antibody at a final concentration was quantified by fluorometry using a QuantiFluor RNA System (Promega) and Superscript III Reverse Transcriptase (Invitrogen, 18080093).

to determine the fold-enrichment of respective marker genes in immunoprecipitated RNA compared with input purity, DNA oligonucleotides were used with FastStart Universal SYBR Green Master (Roche, 4913914001) and comparative Cq method. Samples were considered to be specific if immunoprecipitated RNA exhibited correct de-enrichments or enrichments of respective marker genes and if RNA of control samples did not show any selectivity for marker genes. For each assay, two technical replicates were performed and the means were calculated. The RNA levels were normalized to Gapdh mRNA. Quantitative RT–PCR assays were analyzed using the software StepOne.

The assessment of fold-enrichments for RNA obtained from Vip-cre samples was performed using commercial Tagman probes from Applied Biosystems: Vip (Mm00680234_m1), Vat (Mm00491438_m1), Vglut1 (Mm00812886_m1) and Glap (Mm01253033_m1).

The following DNA oligonucleotides were used with SYBR Green-based real-time PCR (name and sequence 5’ → 3’ are indicated):

Camk2-F: AGAAGTTCAATGTCAGGAGG
Camk2-R: CAGAAGATCTCTTCCACACCA
Gad65-F: GTACTCTCCAGAGAAGTGACG
Gad65-R: GAAATAGTGAAGGTCTGTG
Gapdh-F: CTTGTCTATTTCTCCTTGTTCA
Gapdh-R: CTCTGTGTGATTGTTGGAGAG
Glap-R: AGTTCTCGGACATCTCTCTCT
Povlb-F: CATTGGAGGAGAGAATCTG
Povlb-R: ATGGAGGATTCATTTCAACC
Ptvs3-F: TGACTGTTGTAGTGAACACCA
Ptvs3-R: CTGCATCTGTCCTCCTCTCC
Rgs14-F: ATGATGGTGGAAGATCTCGTG
Rgs14-R: TTACATCTGTTGACCTCC
Sitt-F: AAGAGGACACGGGGATGCACG
Sitt-R: CGGTTAGTGGAGACGCTCAA
Sst-F: CGTCAGTCTGTCGAGAATGTC
Sst-R: AGTACTGCGCCGATTCTGCT
Tlr2-F: AGATGATGGTGCGCCCTTTG
Tlr2-R: GCCCTGTTCCTTACCACTTTAG
Vgat-F: CGTGCACACGATGCTTCA
Vgat-R: AAGATTGAGGAGAACACCCC
Vglut1-F: ACCCCTGTTACAGATTTAACAC
Vglut1-R: CAGTGAAAGGTCGACGTG
Wsf1-F: CATTACGTCACCAACCC
Wsf1-R: TACCTCACACCCGCTTGGC

Library preparation and Illumina sequencing. For all five neocortical and hippocampal neuronal populations, four biological replicates with RIN > 7.5 were analyzed, resulting in a total of 32 individual samples. Library preparation for all samples was performed with 50 ng of RNA using a TrueSeq PolyA Stranded mRNA Library Prep Kit High Throughput (Illumina, RS-122–2103). Libraries were quality checked on a Fragment Analyzer (Advanced Analytical) using a chip quality check on a Bioanalyzer instrument (Agilent Technologies) using a mRNA Library Prep Kit High Throughput (Illumina, RS-122–2103). Libraries were quality checked on a Fragment Analyzer (Advanced Analytical) using a Standard Sensitivity NGS Fragment Analysis kit (Advanced Analytics, DNF-473), which revealed excellent quality of the libraries (average concentration was 48 ng/µl and average library size was 329 ± 82 base pair). The 32 samples were pooled to equal molarity and the pool was quantified by PicoGreen Fluorometric measurement. The pool was adjusted to 10 µM for clustering on C-Bot (Illumina) and then sequenced Paired-End 101 bases using a HiSeq SBS kit v4 (Illumina, FC-401–4003) on a HiSeq 2500 system. Primary data analysis was performed using the Illumina RTA v.1.18.66.3 and bcl2fastq v.2.0.0.422.

Quality control and RNA-seq pre-processing. The splicing analysis of RNA-seq data was performed in collaboration with the company GenoSplice technology (http://www.genosplice.com). Data quality, reads repartition (for example, for potential ribosomal contamination) and insert size estimation were performed using FastQC, Picard-Tools, Samtools and reseq. Reads were mapped using STAR (v.2.4.0) against the exons defined in the proprietary Mouse FAST DB v2016_1 (http://www.genosplice.com). Data quality, reads repartition (for example, for potential ribosomal contamination) and insert size estimation were performed using FastQC, Picard-Tools, Samtools and reseq. Reads were mapped using STAR (v.2.4.0) against the exons defined in the proprietary Mouse FAST DB v2016_1 (http://www.genosplice.com). Data quality, reads repartition (for example, for potential ribosomal contamination) and insert size estimation were performed using FastQC, Picard-Tools, Samtools and reseq. Reads were mapped using STAR (v.2.4.0) against the exons defined in the proprietary Mouse FAST DB v2016_1 (http://www.genosplice.com). Data quality, reads repartition (for example, for potential ribosomal contamination) and insert size estimation were performed using FastQC, Picard-Tools, Samtools and reseq. Reads were mapped using STAR (v.2.4.0) against the exons defined in the proprietary Mouse FAST DB v2016_1 (http://www.genosplice.com). Data quality, reads repartition (for example, for potential ribosomal contamination) and insert size estimation were performed using FastQC, Picard-Tools, Samtools and reseq. Reads were mapped using STAR (v.2.4.0) against the exons defined in the proprietary Mouse FAST DB v2016_1 (http://www.genosplice.com).
samples. The hippocampal samples were contrasted in all pairwise combinations. A series of additional pairwise contrasts were conducted for comparisons across anatomical brain regions. Results for these contrasts using the Ward test and Benjamin–Hochberg Value adjustment as implemented in DESeq2 are compiled in an Excel workbook (Supplementary Table 1). For the principal component analysis, counts were normalized using the variance stabilizing transform as implemented in DESeq2. For heatmaps and the web app plots, the internal normalization factors of DESeq2 were used to normalize the counts.

Alternative splicing analysis. Analysis at the splicing level was first performed taking into account reads mapping to exonic regions and to exon–exon junctions (exon analysis) to potentially detect new alternative events (that is, without taking into account known alternative events). When mapping to exon–exon junctions, reads were assigned to both exons and were therefore counted twice (the minimum number of nucleotides required to be considered mapped to an exon is seven).

To consider an exon expressed, FPKM values for exons must be greater than 96% of the background FPKM value based on intergenic regions. Only exons expressed in at least three of the four biological replicates of each condition and in at least one of the compared experimental conditions were further analyzed.

For illustrations of the Exon and Transcript diagrams, refer to Supplementary Fig. 5. Briefly, for every expressed exon from expressed genes, a SI (defined as the ratio between read density on the exon of interest (that is, the row number of reads on the exon/exon length in nucleotides) and read density on constitutive exons of the gene; ‘class 2’) was generated, as well as fold-change (log(FC)), calculated by comparing the SI value for one condition to the mean SI value in all conditions considered) and P value (unpaired Student’s t-test). Results were considered statistically significant for P ≤ 0.01 and log(FC) ≥ 1 or ≤ -1.

Analysis at the splicing level was also performed by taking into account known splicing patterns (Pattern analysis) annotated in the FAST DB database (that is, for each gene, all annotated splicing patterns were defined, and a SI was generated from this comparison). The normalized read density for a particular annotated pattern.

For an illustrative cartoon of the Pattern analysis process, refer to Supplementary Fig. 5. All types of alternative events can be analyzed, including alternative TSSs, ALEs, cassette exon, mutually exclusive exons, alternative 5′ donor splice sites, alternative acceptor splice sites, intron retention, internal exon deletion and complex events (corresponding to a mix of several alternative event categories). In Supplementary Figs. 2 and 8, we merged intron retention and internal exon deletion events to one single category (‘intron retention’).

Pattern analysis was performed for every condition; log(FC) of SI against P value (unpaired Student’s t-test) were generated. Results were considered statistically significant for P ≤ 0.01 and log(FC) ≥ 1.

FAST DB database includes annotations of 4,965 microexons (defined as exons that are 3–27 nucleotides long), out of the 268,827 total exons annotated (1.8%). In the neuronal populations analyzed, we identified 4,140 (in the neocortex) and 223 (in the primary somatosensory area (S1) for Camk2δ, Pvlb3 and Vip-positive neuronal cells and from L4 of S1 for the alternation events present in the neocortex). For an illustrative cartoon of the Exon analysis process, refer to Supplementary Fig. 5. All types of alternative events can be analyzed, including alternative TSSs, ALEs, cassette exon, mutually exclusive exons, alternative 5′ donor splice sites, alternative acceptor splice sites, intron retention, internal exon deletion and complex events (corresponding to a mix of several alternative event categories). In Supplementary Figs. 2 and 8, we merged intron retention and internal exon deletion events to one single category (‘intron retention’).

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neurobasal medium (Gibco, 21103–049) containing 2% B27 supplement (Gibco, 17504–044), 1% GlutaMAX supplement (Gibco, 35050–038) and 1% penicillin–streptomycin (Sigma, P4333). All 7 days in vitro, cortical cultures were transfected with 400 ng per well of splicing reporters and Lipofectamine 3000 reagent (ThermoFisher Scientific, L3000008) diluted in opti-MEM medium (Gibco, 31985–062) using a 1:1.5 DNA/Lipofectamine ratio. A total of 20,000 neuroblastoma 2a (Neuro2a) or HEK293T cells (obtained from American Type Culture Collection) were plated in 96-well plates and were kept in DMEM (Sigma, D5796) supplemented with 10% FBS (Gibco, 10270106) and 1% penicillin–streptomycin at 37 °C. After 24 h, cells were transfected using FuGENE HD Transfection reagent (Promega, E2691) with 50 ng of splicing reporter DNA alone or in combination with 50 ng of splicing factor DNA.

RNA isolation, reverse transcription and RT–PCR. After 24 h (for Neuro2a and HEK293T cells) or 48 h (for cortical neurons) post-transfection, cells were lysed with 100 μl or 600 μl, respectively, of RLT buffer from a RNeasy Plus Micro kit (Qiagen, 74004) supplemented with 2-mercaptoethanol (Sigma–Aldrich), and RNA was purified according to the manufacturer’s instructions. A total of 400 ng of RNA was reverse transcribed from all samples using random primers (Promega, C118A) and ImProm-II Reverse Transcriptase (Promega, A3802).

For evaluation of differential reporter processing in different cells and conditions, cDNA amounts and PCR cycle numbers were carefully titrated to ensure linear amplification range. Standard PCR reactions were performed using 5× Firepol Master Mix (Solis BioDyne, 04–11–00125) and DNA oligonucleotides targeting the RFP sequence (to avoid detection of endogenous transcripts) and the last flanking exons. The following DNA oligonucleotides were used for standard PCR (name and sequence 5’→3’ are indicated):

- **RFP_F**: AAGCTGGAACATCACCTCCCA
- **Gabrg2_e10_R**: ATGGTTGTGGATCTGGGACG
- **Grin1_e5_R**: ATCACAGACGCGTACCAT
- **Kcnq2_e14_R**: TCGGGCTGTCATCAAGACTC

**GO analysis.** Analysis of GO terms both for neocortical and hippocampal samples was performed using the statistical overrepresentation test of the Panther classification system (Panther14.1, released 8 March 2019 and 17 April 2019), available on http://pantherdb.org. Genes showing significant differential expression (log2(FC) ≥ 0.6 and ≤–0.6, P ≤ 0.05; base mean for neocortex: all neocortical samples) and genes with significant alternative splicing events (log2(FC) ≥ 1 or ≤–1; P ≤ 0.01 from either exon or Pattern analysis) were analyzed using the GO cellular component annotation dataset and Fisher’s exact test with Benjamini–Hochberg false discovery rate (FDR) correction for multiple testing. Alternative TSS events were excluded to analyze the functional role of alternative splicing programs only. To be considered significant, GO terms must have a minimum number of 10 genes, fold-enrichment ≥2 and FDR ≤0.05. As a background reference list, all genes expressed (see Methods for details of gene expression) in the neocortex for neocortical comparisons or in either cell class of the pairwise comparisons were used. Panther output lists GO terms in a hierarchical organization, enabling identification of super-categories, which were further analyzed. Moreover, only terms significant in at least one neocortical population, hippocampal comparison or across anatomical region were used for heatmap visualization. In Fig. 5a, corresponding fold-changes of the gene expression analysis were incorporated. Redundant term categories were excluded.

**General statistical methods.** Sample sizes were chosen based on previous experiments and literature surveys. No statistical methods were used to predetermined sample sizes. Exclusion criteria used throughout this manuscript were predefimined. There are detailed descriptions in the respective sections of the Methods. Group assignment was defined by genotype, thus, no randomization was necessary. Knowledge of experimental conditions was needed for proper execution of experiments. Therefore, investigators were not blinded during data collection and/or analysis. Appropriate statistical tests were chosen based on sample size. Due to “n” in the analysis, normal distribution and equal variances of measures were not formally tested. Thus, individual data points or measures are presented in the manuscript, and data distribution was assumed to be normal. Sequencing analysis was performed on four animals per genotype exhibiting similar variances. N numbers for in situ hybridizations and RT–PCR assays are indicated in the figures. P-value calculations were performed using Student’s t-tests, Ward tests or one-way analysis of variance (ANOVA) with Tukey’s multiple comparison test. FDR calculations were performed using Benjamini–Hochberg correction.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Detailed analyzed data are included as supplementary material. Raw sequencing data were deposited at GEO (accession code: GSE133291). Differential gene expression and splicing data for individual genes are provided on the freely available SpliceCode website (https://scheiffele-splice.scicore.unibas.ch). All renewable reagents and detailed protocols will be made available upon request.

**Code availability**

Data analysis used standard software packages, which are cited in the Methods. The FastDB database for quantitative splicing analysis is a proprietary database accessible through Genosplice Technology (http://www.genosplice.com/).

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Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted
- Give *P* values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated
- Clearly defined error bars
- State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

- Image acquisition: Zeiss Efficient navigation (ZEN) 2010 (Zeiss)

Data analysis

- Differential gene expression analysis: DESeq2 v1.22.2 (Love, 2014, Genome biology).
- Quantitative splicing analysis: FASTDB is a proprietary database accessible through Genosplice Technology (http://www.genosplice.com; FAST DB v2015_1 database; de la Grange, 2007, BMC Bioinformatics; S. Gandoura, 2013, Hepatol.; E.Wang, 2012, PLoS One).
- Image analysis: Fiji, version 20.0-ev-69/1.52i

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The raw data generated during the current study are available at the GEO repository (GSE133291). The analyzed data is included in the manuscript as supplementary material in excel format and is also provided at the SpliceCode web-site (https://scheiffele-splice.scicore.unibas.ch). Renewable reagents and detailed protocols will be made available upon request.

Field-specific reporting

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☐ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample sizes were chosen based on previous experiments and literature surveys. No statistical methods were used to pre-determine sample sizes |
| Data exclusions | Exclusion criteria were pre-established. These included assessments of the quality of the RNA prior to deep-sequencing analysis (RNA integrity, appropriate enrichment of respective markers) and quality control of sequencing libraries. |
| Replication | Sequencing data has been validated by RT-PCR resulting in a validation rate > 90% (20 out of 22 splicing events tested) and In-situ hybridization, Supplementary Fig.6, Supplementary Fig.10 |
| Randomization | Group assignment was defined by genotype, thus, no randomization was necessary. |
| Blinding | Data collection and analysis were not performed blind to the conditions of the experiments. |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
| n/a | Unique biological materials |
| ☒ | Antibodies |
| ☒ | Eukaryotic cell lines |
| ☒ | Palaeontology |
| ☒ | Animals and other organisms |
| ☒ | Human research participants |

Methods

| n/a | Involved in the study |
| ☒ | ChIP-seq |
| ☒ | Flow cytometry |
| ☒ | MRI-based neuroimaging |

Antibodies

Antibodies used

The antibody used in this study is a rat anti-HA antibody from Roche catalogue number #11867431001, clone 3F10 monoclonal, dilution 1:1000

Validation

The rat anti-HA antibody from Roche was tested for immuno-histochemistry on mouse brain sections which expressed an HA tag in defined subpopulations of cells and mouse brain sections from littermates devoid of the HA tag. Immuno-positive signals could only be detected in brain sections containing the HA tag. These quality controls are not displayed in the manuscript. The antibody was tested by the manufacturer using Western Blot and detection of recombinant HA protein.
### Eukaryotic cell lines

**Policy information about cell lines**

| Cell line source(s)                  | ATCC (American Type Culture Collection) for HEK239T and Neuroblastoma 2a cell lines |
|-------------------------------------|--------------------------------------------------------------------------------------|
| Authentication                      | Cell lines from ATCC have been thoroughly tested and authenticated by the company. The identity of the cell lines was confirmed using morphology, karyotyping and PCR based approaches to rule out intra- and interspecies contamination |
| Mycoplasma contamination             | Cell lines were checked for mycoplasma contamination by RT-PCR and tested negative for contamination. |
| Commonly misidentified lines (See ICLAC register) | No commonly misidentified lines were used in this study |

### Animals and other organisms

**Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research**

| Laboratory animals | This study used laboratory mice which were maintained in C57Bl6/J background and obtained from Jackson Laboratories. Stock # 011029, 017320, 013044, 005359, 006474, 031628, 009613. Both male and female animals were used. The age ranged from p25 to p42 |
| Wild animals        | This study did not involve wild animals |
| Field-collected samples | This study did not involve field-collected samples |