Enhancers active in dopamine neurons are a primary link between genetic variation and neuropsychiatric disease

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Enhancers function as DNA logic gates and may control specialized functions of billions of neurons. Here we show a tailored program of noncoding genome elements active in situ in physiologically distinct dopamine neurons of the human brain. We found 71,022 transcribed noncoding elements, many of which were consistent with active enhancers and with regulatory mechanisms in zebrafish and mouse brains. Genetic variants associated with schizophrenia, addiction, and Parkinson's disease were enriched in these elements. Expression quantitative trait locus analysis revealed that Parkinson's disease-associated variants on chromosome 17q21 cis-regulate the expression of an enhancer RNA in dopamine neurons. This study shows that enhancers in dopamine neurons link genetic variation to neuropsychiatric traits.

To date the majority of disease- and trait-associated variants emerging from genome-wide association studies (GWAS) of neurologic and psychiatric diseases lie within nonprotein coding sequences. Several lines of evidence suggest that a proportion of such variants are involved in transcriptional regulatory mechanisms, including modulation of enhancer elements. Many regulatory elements are cell-type-preferential1,2, and therefore sequence variants with functional consequences are expected to manifest their effects more strongly in the cell type(s) most relevant to a specific disease phenotype.

Here we focused on systematically identifying all noncoding regulatory elements transcriptionally active in a morphologically, functionally, and biochemically distinct neuronal archetype: dopamine neurons of the substantia nigra pars compacta in human midbrain. We hypothesized that genetic variation associated with diseases involving dopaminergic neurotransmission exerts its effects through modulation of enhancers functionally active in this particular type of neurons. Perturbations of the dopaminergic system are important in the pathogenesis and treatment responses of many increasingly prevalent complex genetic diseases, including Parkinson's disease (PD), which affects 0.5 million people, schizophrenia, which affects 2.2 million people, and addiction, which affects 23.5 million people (all numbers are for the United States alone). In healthy people, these dopaminergic neurons shape how we conduct our everyday lives, encoding activities related to motivation and reward. Signals from these neurons to the striatum have a profound impact on action learning and automatic movements, while projections to hippocampus and prefrontal cortex influence memories and behavior.

Our analysis is powered by an integrated hardware–software solution for comprehensively detecting noncoding transcription in one single and minuscule RNA sample and mapping the variation in noncoding transcription to genetic variation within dopamine neurons across multiple individuals. This method combines the base-pair resolution and a comprehensive genome-wide view afforded by ultradeep, total RNA-sequencing with the positional and cytoarchitectural precision afforded by traditional light microscopy.

Results

Identification of noncoding elements actively transcribed in dopamine neurons of human brain. To systematically identify noncoding elements actively transcribed in dopamine neurons of human brain, we used laser-capture microdissection total RNA-sequencing (lcRNAseq). Beyond traditional mRNA sequencing,
all polyadenylated and non-polyadenylated transcripts were ultradeeply sequenced using ribodepleted RNAs from ~40,400 neurons laser-captured from 99 human postmortem brains and seven non-neuronal cell-type samples with an average of 178 million reads per sample, yielding 2.0 × 10^8 pair-ended RNA-seq reads (Supplementary Table 1). Melanized neurons from the midbrain substantia nigra pars compacta of 86 high-quality human brains (dopamine neurons), pyramidal neurons from layers V/VI of the middle temporal cortex of ten brains, and pyramidal neurons from the primary motor cortex of three brains (pyramidal neurons) were laser-captured as described8–10 (Fig. 1a). Human fibroblasts from four individuals and peripheral blood mononuclear white cells (non-neuronal cells) from three individuals were analyzed in the same pipeline (Supplementary Figs. 1a and 2 and Supplementary Table 2). Cumulatively, we found that at least 64.4% of the human genome was transcribed to produce detectable RNAs in dopamine neurons of the human brain (Fig. 1b and Methods), consistent with observations from the Encyclopedia of DNA Elements project (ENCODE) in cultured cells11. More than half of these reads (54.7%) mapped to intergenic or intronic regions (Fig. 1c), indicating a massive hidden layer of active noncoding transcription in human brain neurons.

Enhancer RNA (eRNA) expression is a marker for active enhancers12–14 and can be used to estimate enhancers active in a particular cell type at given time15. Genetic enhancer elements control the cell-type-specific activation of gene expression. We designed a sophisticated method to systematically identify noncoding elements, including known and novel candidate enhancers that are significantly expressed in dopamine neurons, pyramidal neurons, and non-neuronal cells, using a stringent six-step filter (Fig. 1d and see Methods for details). We required aggregated reads for each cell type to achieve local peak read densities (‘summits’) with detection \( P \leq 0.05 \) compared to randomly sampled background; without overlap with exons from annotated genes and transcription start site-proximal regions; with a minimal element length of 100 bp; and without splicing junction reads (to avoid multiexon noncoding RNAs). We then rigorously determined the statistical significance of each of these candidate transcribed noncoding elements across multiple independent samples of the same cell type (for example, across 86 independent samples for dopamine neurons) with a family-wise adjusted \( P \leq 0.05 \) taken as evidence of statistically significant expression.

We discovered 71,022, 37,007, and 19,690 transcribed noncoding elements (TNEs) in dopamine neurons, pyramidal neurons, and non-neuronal cells, respectively, with detection \( P \) values equal or better than the Bonferroni-corrected significance thresholds of \( 7.0 \times 10^{-7}, 5.1 \times 10^{-7}, \) and \( 6.6 \times 10^{-7} \) for each of the three cell types, respectively (Supplementary Table 3). The length distribution of TNEs peaked around 400 bp (Supplementary Fig. 3a), consistent with that of the eRNAs previously reported by FANTOM513 and of activity-regulated eRNAs found in mouse cortical neurons14. Unlike promoter regions, TNEs showed a GC content distribution similar to that of eRNAs previously reported by FANTOM513 and of activity-regulated eRNAs found in mouse cortical neurons14.
to that of random genomic background regions, and this is inconsistent with PCR bias (Supplementary Fig. 3b). The vast majority of TNEs (92%) localized to intronic regions (Supplementary Fig. 3c) and tended to be positionally biased toward the 5’ end of gene body, a pattern opposite to that of partial RNA degradation, which preferentially degrades 5’ ends (Supplementary Fig. 3d). TNEs accounted for 31.42% and 32.35% of reads transcribed in dopamine and pyramidal neurons, respectively, compared to 21.08% in peripheral cells; and 26.38% of dopamine neuron TNEs were also presented in pyramidal neurons (Fig. 1e; Fisher’s exact test $P < 2.2 \times 10^{-16}$, odds ratio $= 3.22$), but only 7.85% in peripheral cells. Subprograms of protein-coding mRNAs and noncoding RNAs (ncRNAs) expressed in dopamine neurons, pyramidal neurons, and non-neuronal cells were also characterized (Supplementary Fig. 4, Supplementary Table 4, and Methods).

**TNEs identify putative enhancers active in dopamine neurons.** Of the 71,022 TNEs active in dopamine neurons, 23,625 (33%) coincided with enhancers defined by one or more genomic or epigenetic features (Fig. 2 and see Methods). These features included DNase I hypersensitivity sites (DHS)\(^2\), characteristic histone modifications (such as high H3K27ac, high H3K4me1, and low H3K4me3)\(^3\), capped analysis of gene expression (CAGE)\(^4\)-defined enhancers, transcriptional coactivator P300\(^5\) binding sites, transcription factor ‘hotspots’\(^6\), and sequence conservation\(^7\). Of the 71,022 TNEs, 20,505 coincided with chromatin-state-defined putative active enhancers from Roadmap Epigenomics\(^8\) and 1,212 TNEs coincided with CAGE-defined putative active enhancers\(^9\). The overlap was significantly higher than expected by chance alone ($P < 2.2 \times 10^{-16}$ by permutation test; Supplementary Table 5).

We performed two experiments to directly benchmark TNE to putative enhancers predicted by two other methods applied to the same source (Fig. 2b). Of the TNEs called by our pipeline in the human cortex dataset from PsychENCODE\(^10\), 44.1% (14,904 of the 33,762 TNEs) overlapped with a strong transposase-accessible chromatin assay sequencing (ATAC-seq) peak (which maps chromatin accessibility\(^11\)) identified in the same samples (Fig. 2b). This was a significantly higher than expected by chance ($P < 2.2 \times 10^{-16}$ by permutation test). In SK-N-SH (human neuroblastoma cell line) cells, 21.7% of called TNEs (11,465 of 52,733) overlapped with putative enhancer features (Fig. 2b; for example, H3K27ac, H3K4me3, transcriptional regulator CTCCT-binding factor (CTCF) chromatin immunoprecipitation sequencing (ChIP-seq), P300 ChIP-seq, DNase I hypersensitivity, and transcription factor ‘hotspots’ delineated by ENCODE in this cell line ($P < 2.2 \times 10^{-16}$ by permutation test), similarly to the 25% overlap previously reported between CAGE-defined and chromatin state-predicted putative enhancers\(^12\).

We grouped 71,022 dopamine TNEs into three classes according to the presence or absence of supporting features (see Methods). Specifically, 11,835 TNEs coincided with multiple supportive features (designated class I TNEs), i.e., a known DHS site plus at least one of five additional external features (enhancer chromatin state (chromHMM), CAGE-enhancer, P300 peak, transcription factor binding sites hotspot, and highly conserved noncoding elements between human and zebrafish; Fig. 2c,d). A second set of 11,790 TNEs was supported by at least one of the five external features, but lacked additional DHS evidence (designated class II TNEs; Fig. 2c,d). A third set of 47,397 TNEs had no previously reported supporting external features (termed class III TNEs; Fig. 2c,d). Bidirectional transcription of select dopamine TNEs was seen using CAGE in substantia nigra of four of the same brains used for lcrRNAseq (Supplementary Fig. 5a). Moreover, transcription factor binding sites were enriched in TNE sites, based on in silico analysis of ChIP-seq peaks and motif scanning (Supplementary Fig. 5b–d and Supplementary Note).

**Replication of TNEs in independent cohorts.** We replicated pyramidal neuron TNEs in three independent cohorts representing 36, 498, and 795 human brain samples, respectively (Fig. 3a), and additionally confirmed select TNEs with two secondary methods (Fig. 3b and Supplementary Fig. 5a). Of the 37,007 pyramidal neuron TNEs discovered, 34,077 (92.1%) were replicated in an independent cohort of pyramidal neuron laser-captured from layer V/VI of 36 new human autopsy brains (Fig. 3a). We identified 14,679 (39.7%) and 10,718 (29%) of 37,007 pyramidal neuron TNEs from ribodepleted total RNA-seq data of frontal cortex (PsychENCODE\(^10\)) and four cortex areas (Accelerating Medicines Partnership–Alzheimer’s Disease Consortium (AMP-AD)), respectively (Fig. 3a). Select brain cell-type-specific TNEs were confirmed with a secondary method, quantitative PCR (qPCR), in laser-captured dopamine neurons (Fig. 3b). As expected, qPCR analysis of control samples lacking template or reverse transcriptase showed no expression of TNE. Finally, we confirmed a subset of dopamine neuron TNEs by performing CAGE on four substantia nigra homogenate samples (Supplementary Fig. 5a and Methods).

**TNE signatures accurately cluster dopamine and pyramidal neurons.** A majority (57.5%; 40,846 of 71,022) of the detected TNEs were exclusively expressed in human dopamine neurons. They were not detected in pyramidal neurons or non-neuronal cells. Thirty-nine percent (14,487 of 37,007) of pyramidal neuron TNEs were exclusive to this cell type; 64% (12,601 of 19,690) of non-neuronal TNEs were exclusively expressed in non-neuronal cells (Fig. 1c). A signature based on cell-type-exclusive TNEs clustered 106 individual samples with 99.1% accuracy (Fig. 3c), similar to the classification accuracy afforded by mRNAs and ncRNAs (Supplementary Fig. 4). Normalized counts for the 100 most-abundant exclusive TNEs in each cell type are visualized in Fig. 3d. Cell-type-preferential expression of three dopamine neuron-exclusive TNEs, three pyramidal neuron-exclusive TNEs, and one TNE common to both dopamine and pyramidal neurons (in intron 4 of the PD gene SNCA\(^23\)) was confirmed by qPCR in additon to lcrRNAseq (Fig. 3b and Supplementary Fig. 6b). These TNEs were in close proximity to histone marks typical of active enhancers\(^24\) as well as multiple transcription factor occupancy sites\(^25\) (Fig. 3b).

**In vivo validation of TNE enhancer activity in zebrafish, mice, and neuronal cells (Fig. 4).** To determine whether TNEs can function as enhancers, we tested 15 TNEs (Supplementary Table 6) in vitro in human SK-N-MC neuroblastoma cells and non-neuronal HeLa cells. TNE sequences were inserted into a modified pGL4.10 vector (as in ref.\(^16\)), for example, upstream of an EF1a basal promoter separated by a synthetic poly-A signal or transcriptional pause site to avoid promoter effects. Eleven of the 15 TNEs (73%) significantly increased reporter activity in neuronal cells compared to control inserts representing random background sites (Fig. 4b). Eight TNEs induced more than a two-fold increase in reporter signal (Fig. 4b), and all but one TNE exhibited considerably higher enhancer activity in the neuronal cells compared to HeLa cells (Fig. 4b).

**VMP1-TNE (chr17:57,863,430–57,864,538) is located in intron 7 of the human VMP1 gene, a key regulator of autophagy. The VMP1-TNE site is evolutionarily conserved among vertebrates and actively transcribed in human brain dopamine neurons, pyramidal neurons, and non-neuronal cells. VMP1-TNE was a class I TNE with a bimodal distribution of RNA-seq reads (centered on the DHS peak; Fig. 4a), bidirectional CAGE signal (Fig. 4a), occupancy by 90 TFs (Fig. 4a), and high levels of H3K4me1 and H3K27ac (Fig. 4a), and it was predicted as putative enhancer by ChromHMM\(^26\) in Roadmap Epigenomics\(^27\). It was highly active in neuroblastoma and HeLa cells in culture (Fig. 4b). To assess the activity of VMP1-TNE in vivo, transient transgenic reporter assays were carried out in zebrafish embryos. The PCR-amplified sequence was cloned upstream of...
zebrafish gata2 minimal promoter, linked to an mRuby2 reporter gene in a modified pDB896 vector. A similarly sized sequence amplified from a nonconserved intergenic region with very low or no signal for enhancer marks was used to generate a control construct. Embryos injected with Has.VMP1-TNE:gata2:mRuby2 (Fig. 4c–g) reporter construct showed reproducible enrichment.
of enhancer activity in a specific subset of telencephalic neurons near the eyes and in cardiac cells proximal to the atrioventricular canal compared to embryos carrying control construct (Has. control:gata2:mRuby2; Fig. 4c–g and Supplementary Table 7), consistent with the expression pattern of miR-21 (http://zfin.org/), the putative target gene in the synteny block as suggested by comparative genomics (Supplementary Fig. 7).

The VISTA consortium has established one of the largest repositories of in vivo enhancer screens during mouse development. Sequences overlapping with 96 dopamine neuron TNEs were evaluated by VISTA, 63 (65.6%) of which were positive enhancers in vivo in mice, considerably more than expected by chance alone \( (P = 3.91 \times 10^{-3} \) by Fisher’s exact test; Fig. 4h). The enrichment for VISTA-validated enhancers was similar for class I and III TNEs.
(Supplementary Fig. 6c). Notably, 35 of these 63 (55.6%) VISTA-validated TNEs drove reporter gene expression in neuronal tissues, particularly midbrain, hindbrain, and the neural tube. For example, a neuron-specific TNE located in the intron of autism susceptibility candidate 2 gene (AUTS2) enhanced reporter activity specifically in the midbrain in 11 of 15 mouse embryos tested\(^{(28)}\) (Fig. 4i and Supplementary Fig. 6d). In comparison, of 31 exclusively non-neuronal TNE evaluated by VISTA, 14 (45%) were positive enhancers, and only 9 (29%) were active in neuronal tissues. Collectively, these test cases show that select TNE sites enhance reporter gene expression in human neuronal cells and in neurons in the brains of zebrafish and in mice.

Variants associated with diseases of the dopamine system are over-represented in TNE actively transcribed in dopamine neurons. GWAS variants for 61 diseases and traits were significantly enriched within noncoding elements functional in dopamine neurons with \(P\) values below the Bonferroni-corrected significance threshold of \(9.64 \times 10^{-8}\) by Fisher’s exact test (for example, 0.01 divided by 1,037, the total number of traits in the NHGRI GWAS catalog\(^{(29)}\)) compared to random background (Fig. 5a and Methods). By contrast, only 43 traits were significantly enriched in promoters, 11 of them in exons (Fig. 5a). Consistent with our hypothesis, variants associated with 11 diseases and medications perturbing the dopamine system were significantly enriched in dopamine neuron TNE sites (Fig. 5a.b). These included variants associated with schizophrenia \((P = 1.75 \times 10^{-8})\), PD \((P = 5.05 \times 10^{-8})\), addiction \((P = 1.33 \times 10^{-8})\), and bipolar disorder \((P = 5.05 \times 10^{-8})\). Moreover, pharmacogenetic variants associated with response to antipsychotics were enriched in these TNE sites \((P = 4.39 \times 10^{-10})\). Classical antipsychotics are dopamine receptor antagonists that are the standard treatment for schizophrenia. Variants associated with response to iloperidone, a specific antipsychotic medication for schizophrenia, were also enriched \((P = 1.94 \times 10^{-8})\). Variants associated with response to the dopamine reuptake inhibitor methylphenidate (used to treat attention deficit hyperactivity disorder) were enriched in dopamine neuron TNE sites \((P = 8.74 \times 10^{-8})\). By contrast, none of these trait variants were enriched in promoters or exons. Notably, traits relating to sleep phenotypes, which are modulated by dopamine neurons (for example, refs.\(^{(28,29)}\)) and perturbed in PD\(^{(6)}\), were highly enriched in these TNE sites \((P = 2.6 \times 10^{-35})\; (Fig. 5a.b). Strikingly, cardiovascular traits (Fig. 5a.b); diseases and traits clustering around obesity, weight, and diabetes (Fig. 5a.b); and brain-volume-related traits (Fig. 5a.b) were also over-represented in dopamine neuron TNEs compared to random genomic background. The enrichment density for dopamine system traits was similar for each of the three TNE classes (Supplementary Fig. 8a).

Dopamine neuron TNEs harbor a higher density of GWAS variants linked to traits of the dopamine system than enhancer predictions without cell-type-specificity. GWAS single-nucleotide polymorphism (SNP) density analyses showed a higher density of GWAS variants for dopamine system traits in TNE active in midbrain dopamine neurons compared to FANTOM5-predicted and ChromHMM-predicted putative enhancers, exons, promoters, introns, intergenic regions, and length-matched random regions (Supplementary Fig. 9).

Expression quantitative trait locus analysis reveals transcribed noncoding elements in synapse genes as main cell-autonomous effectors of \(cis\)-acting genetic variation. Expression quantitative trait locus (eQTL) analysis for TNEs, ncRNAs, and mRNAs was—for the first time to our knowledge—performed across cell-type-specific transcriptomes from 84 human brains (Fig. 5c). We measured or imputed 4,283,750 SNPs and associated them with normalized TNE expression using Matrix eQTL\(^{(13)}\) (see Methods). Of these, 8,676 \(cis\)-acting TNE eQTLs achieved a false discovery rate of less than or equal to 0.05, comprising 3,461 unique expression-associated SNPs (eSNPs) and 151 unique TNEs (Fig. 5c). On average, 23 eSNPs were associated with expression changes in one TNE. Furthermore, 3,381 ncRNA eQTLs were significant \((FDR \leq 0.05)\), comprising combinations of 3,320 unique eSNPs and 52 unique expressed ncRNA genes (Fig. 5c and Supplementary Fig. 10). By contrast only 1,150 mRNA eQTLs reached statistical significance \((FDR \leq 0.05)\), comprising combinations of 676 unique eSNPs and 46 unique associated expressed protein-coding genes (Fig. 5c and Supplementary Fig. 10).

These 151 \(cis\)-regulated TNEs physically localized to introns of 102 host genes. These host genes were highly enriched in Gene Ontology (GO) terms related to synapse function \((P < 4.79 \times 10^{-7}\) by enrichment analysis using the hypergeometric test; see Methods and see Supplementary Table 8 for full results) and in medical subject heading terms for brain disorders with \(P = 5.1 \times 10^{-10}\) (Supplementary Table 9). Mutations of several of these synapse-related host genes can cause abnormal brain development and function (Supplementary Fig. 10 and Supplementary Note). Taken together, this gene-regulatory analysis indicates that genetic variation is linked to variation in the activity of putative enhancers in synapse genes, including several loci linked to Mendelian brain disorders.

PD-associated variants \(cis\)-regulate a noncoding element in the \(KANSL1\) gene. Leveraging 495,085 SNPs associated with one or more of 1,037 human diseases or traits \((19,188\) disease-associated SNPs from the NHGRI-EBI GWAS catalog\(^{(29)}\), extended via imputation of proxy SNPs with \(r^2 \geq 0.8\)), we identified 1,989 disease-associated SNPs that influence expression of 19 TNEs, 4 ncRNAs, and 5 mRNAs in \(cis\). To distinguish coincidental co-localizations of GWAS and eQTL associations, we used regulatory trait concordance (RTC) scores\(^{(24)}\), which assess whether a \(cis\)-eQTL and a trait association are tagging the same underlying functional effect. Applying a stringent RTC threshold of 0.85, we identified 23 disease-associated TNE eQTLs for which the trait and TNE expression associations may be tagging the same effect in dopamine neurons (Fig. 5c and Supplementary Table 10). Of these, 17 disease-associated eQTLs were identified for ncRNAs and 1 for mRNAs.

Eight of these 23 TNE-eQTLs linked PD-associated variants to a putative eRNA expressed from intron 2 of the \(KANSL1\) gene with \(P\) values as low as \(1.57 \times 10^{-7}\) (Supplementary Table 10). The corresponding RTC scores were 0.91–1.00, indicating that the GWAS-derived disease variants explain the eQTL observation. Six of the eight PD-associated eSNPs mapped to the exact same 712,000-bp-long linkage disequilibrium (LD) block on chromosome 17q21 (here termed LD2; Fig. 5d) and were significantly associated with upregulation of the same \(KANSL1\)-TNE1 in carriers of risk alleles \((6.46 \times 10^{-7}\)). Two additional eSNPs mapped to a nearby LD block (LD3; Fig. 5d). Conditional eQTL analysis adjusting for the lead GWAS variant rs17649553 suggested that some eSNPs in LD2 and one in LD3 might carry an independent signal (Methods, Supplementary Fig. 11, and Supplementary Table 11). Chromosome 17q21 is the second-most-important GWAS peak for sporadic PD after \(SNCA\) and unequivocally associated with susceptibility for PD, with \(P\) values as low as \(2.23 \times 10^{-48}\) in a meta-GWAS of more than 100,000 cases and controls\(^{(15)}\). There is precedent that copy-number variation in the \(KANSL1\) locus causally impacts brain function, as microdeletions of the locus cause Koolen de Vries syndrome, a neurological disease with severe learning disability and developmental delay. In addition to upregulating the \(KANSL1\)-TNE, the same PD-associated variants in LD2 (but not those localized to LD3) were associated with downregulation of an expressed pseudogene, \(LRRC37A4P\) \((P = 2.36 \times 10^{-7}\); Supplementary Table 10). \(LRRC37A4P\) is localized near \(KANSL1\) under the chromosome 17q21 GWAS peak. By contrast, eQTL associations for \(MAPT\)
**Fig. 4 | In vivo validation of TNE enhancer activity in zebrafish, mice, and neuronal cells.**

**a.** VMP1-TNE in intron 7 of human VMP1. This evolutionary conserved TNE is supported by classical epigenetic features: a putative active enhancer including open chromatin (DNase)20, high levels of H3K4me1 and H3K27ac25, and bidirectional CAGE signal13.

**b.** Enhancer reporter assays for TNE-defined putative enhancers in HeLa S3 (cyan) and SK-N-MC neuroblastoma line (magenta) cells. TNE regions are labeled Pxxx (see Supplementary Note for details); C001 and C002, enhancers from FANTOM5 (positive controls)13; R001, random genomic background region (negative control); n = 4 transfections were independently performed and analyzed for each TNE in HeLa S3 and another n = 4 independent transfections in SK-N-MC cells. *P < 0.05; **P < 0.01; ***P < 0.001; two-tailed Student’s t test. Box plots as in Fig. 3. c–g. Group view of embryos injected with control:gata2:mRuby2 and VMP1-TNE:gata2:mRuby2. Enhanced reporter activity was observed in the embryos injected with VMP1-TNE-containing reporter construct (d) compared to the control element (e). In addition, embryos injected with VMP1-TNE:gata2:mRuby2 (d) show tissue-specific reporter expression in a group of telencephalic neurons in proximity to the eye (arrows) and atrioventricular canal (arrowheads). Background (ectopic) activity (stars) predominantly in skin yolk muscle and autofluorescence from blood and eye pigmentation (stars) was observed in both VMP1-TNE:gata2:mRuby2- and control:gata2:mRuby2-injected embryos. Scale bars, 200 µm. (e) A brightfield reference image of the embryo regions shown in f.g. (f.g) High-magnification view on the head and heart region of ETmef2:gfp transgenic embryos injected with control:gata2:mRuby2 and VMP1-TNE:gata2:mRuby2. GFP reporter expression in these embryos was used as marker for the heart ventricle (dashed line). Expression in the telencephalic neurons (arrows) and atrioventricular canal (arrow heads) in VMP1-TNE:gata2:mRuby2 injected embryos can be seen. Stars, ectopic activity. The experiment was repeated independently with similar results four times, with 478 and 408 embryos screened in total for VMP1-TNE and control constructs, respectively (see Supplementary Table 7 for details). Scale bars, 100 µm.

**h.** Putative enhancers evaluated in mice by VISTA28. Of 1,789 putative enhancers tested in mice by VISTA (left two bars), 929 (52%) were active in mice. By contrast, 63 (66%) of 96 TNE-defined putative enhancers were found to be active enhancers in mice (*P = 3.91 × 10⁻³, hypergeometric test).

**i.** Reporter activity of a neuron-specific intronic TNE located in the AUTS2 gene is seen in the midbrain (red insert) and neuronal tube (blue insert) of mouse embryonic day (E) 11.5 embryos by VISTA28. Embryos have an average crown–rump length of 6 mm.
mRNA, a biological candidate in this region, did not reach genome-wide significance (Fig. 5c–g). The inverse eQTL relations between the lead GWAS-derived SNP, rs17649553, and KANSL1-TNE1 and LRRC37A4P, respectively, were confirmed by a second method, cell-type-specific qPCR (Supplementary Fig. 12a). Moreover, this association was independently replicated in a second cohort of neurons laser-captured from 31 high-quality control brains (Supplementary Fig. 12b and Supplementary Table 12). Third, the rs17649553—LRRC37A4P eQTL association was further confirmed in 56 substantial nigra and 96 frontal cortex samples from the Genotype-Tissue Expression Consortium (GTEx; Supplementary Fig. 12c,d), which used a poly-A+ selection protocol that does not allow for assaying KANSL1-TNE1 RNA.

Discussion

eRNA expression is a feature of active enhancers13–14 and can be used as a marker to estimate their activity in a particular cell type15. Genome elements with enhancer chromatin marks that are transcribed into eRNAs have substantially higher validation rates in vitro enhancer assays than enhancers defined exclusively by chromatin states16. Moreover, in transgenic mouse reporter assays, over half of putative enhancers identified on the basis of deep RNA-seq data functioned as enhancers with reproducible activity in the predicted tissue16. Many chromatin-defined enhancers are not regulatorily active in a particular cellular state, but may be active in other cells17 or are premarked for fast regulatory activity upon stimulation18.

We showed a highly specific program of enhancer elements that are actively transcribed in physiologically and morphologically distinct, disease-relevant dopamine and pyramidal neurons, in situ, in human brains. Nearly two-thirds (64.4%) of the genome were not regulatorily active in a particular cellular state, but may be active in other cells or are premarked for fast regulatory activity upon stimulation19.

Putative enhancers active in dopamine neurons link genetic variation to neuropsychiatric disease.

Fig. 5 | Putative enhancers active in dopamine neurons link genetic variation to neuropsychiatric disease. a. GWAS diseases and traits with variants significantly enriched in dopamine TNEs, exons, promoters, and random background regions. Variants for 61 diseases and traits were enriched within TNE-defined putative active enhancers in dopamine neurons with P values below the Bonferroni-corrected significance threshold of 9.64×10−5 by one-sided Fisher’s exact test compared to 71,022 random genomic background regions (see Methods). The largest share of traits (n=11) enriched within putative active enhancers clustered around perturbations of the dopamine system (pink). By contrast, only 43 traits were enriched in promoters (including two involving the dopamine system), 11 in exons, and none in random background regions. b. Diseases and traits significantly enriched in TNE-defined putative enhancers in dopamine neurons. Variants associated with eleven diseases or medications perturbing the dopamine system (horizontal pink bars) were dramatically over-localized in dopamine neuron TNEs. The number of disease-variants colocalizing to dopamine TNEs for each trait as well as odds ratios (in parenthesis) are shown next to each bar. X axis, P values by one-sided Fisher’s exact test (−log10 scale); y axis, diseases and traits. ADHD, attention deficit hyperactivity disorder; ASD, autism spectrum disorder; MDD, major depressive disorder; ANCA, antineutrophil cytoplasmic antibody; ALL, acute lymphoblastic leukemia; COPD, chronic obstructive pulmonary disorder; QRS duration, a feature on an electrocardiogram. c. eQTL analysis reveals transcribed noncoding elements in synapse genes as main cell-autonomous effectors of cis-acting genetic variation in dopamine neurons. Notably, the number of TNE eQTLs greatly surpassed the number of mRNA eQTLs and ncRNA eQTLs identified. d. The second most significant GWAS locus for sporadic PD is located on chromosome 17q21. This locus shows unequivocal evidence for association with PD. The regulated gene has not been established, but MAPT has been commonly assumed to be the

regulatory function in zebrafish and mouse neurons. We provided mechanistic evidence that some of these elements function as enhancers of transcription in zebrafish brain, in the midbrain of mice, and in human cultured neuronal cells using genetics and reporter assays. Moreover, multiple independent lines of evidence—including chromatin state, CAGE expression, and transcription factor binding analyses—support the view that these transcribed noncoding elements are putative enhancers specifically active in dopamine neurons.

Variants associated with 11 diseases or medications perturbing the dopamine system were enriched in dopamine neuron-specific TNE sites, much more so than in promoters and or exons (Fig. 5a,b). Risk alleles associated with major disorders of dopaminergic neurotransmission, schizophrenia, PD, addiction, and bipolar disorder overlocalized to active TNE sites. Compellingly, even pharmacogenetic variants linked to treatment response were enriched in active enhancers. These observations suggest that GWAS variants might modulate enhancers active in dopamine neurons and thereby regulate the transcriptional program underlying susceptibility for these neuropsychiatric diseases. Finally, risk alleles associated with sleep-related phenotypes were enriched in TNE sites (P=2.6×10−20). Indeed, dopamine neurons have a role in sleep regulation24,25, and REM sleep behavior disorder is an early sign of PD26.

eQTL analysis for putative eRNAs was performed across cell-type-specific transcriptomes from 84 human brains (Fig. 5c). We thereby uncovered transcribed noncoding elements in synapse genes as a main cell-autonomous effector of cis-acting genetic variation in dopamine neurons. Notably, the number of TNE eQTLs greatly surpassed the number of mRNA eQTLs and ncRNA eQTLs identified.

The second most significant GWAS locus for sporadic PD is located on chromosome 17q21. This locus shows unequivocal evidence for association with PD. The regulated gene has not been established, but MAPT has been commonly assumed to be the...
prime candidate. Using eQTL analysis, we provide striking evidence pointing at regulation of a putative eRNA expressed from intron 2 of the KANSL1 gene as a gene-regulatory mechanism for this susceptibility locus. The KANSL1 locus is important for normal brain function. Microdeletions cause Koolen de Vries syndrome, a neurological disease with severe learning disability and developmental delay. The KANSL1-TNE1 eQTL association was confirmed by cell-type-specific qPCR and replicated in an independent cohort. By contrast, eQTL associations for MAPT did not reach statistical significance in dopamine neurons (P = 0.32). Long-read sequencing and larger datasets will be required to comprehensively illuminate the relation between structural variation and transcriptional function in this complex locus.

The KANSL1-TNE1 eQTL appears to be a 'super-eQTL' of variants associated with eight dopaminergic, radiographic, pulmonary, and dermatologic traits all localized to the same LD2 block on chromosome 17q21 and all associated with KANSL1-TNE1 upregulation (Fig. 5c). Six of these seemingly disparate traits are clinically implicated in multisystem features of PD. Progressive supranuclear palsy (trait 2) leads to neurodegeneration of dopamine neurons (Fig. 5c and Supplementary Table 10). Men with early-onset male pattern baldness (trait 3) have a 28% higher risk of developing PD. Genetic variants for intracranial volume (traits 4 and 5) are related to PD, and PD patients are prone to reduced bone mineral density (trait 6). Thus, PD and seven clinically related traits with variants localizing to an LD block on chromosome 17q21...
are associated with KANSL1-TNE1 expression through a uniform gene-regulatory mechanism.

This study was powered by innovations both in wet and dry lab methods and provides an online resource of mRNAs, ncRNAs, and TNE expression in dopamine and pyramidal neurons, as well as dopamine neuron-specific mRNA, ncRNA, and TNE eQTLs (BRAINcode, http://www.humanbraincode.org). Our method allows detection of the full complement of mRNAs, ncRNAs, and active enhancers in a single and minuscule RNA sample and combines the base-pair resolution and a comprehensive genome-wide view afforded by ultradepth total RNA-sequencing with the positional and cytoarchitectural information afforded by traditional light microscopy. It can be transferred to other morphologically or regionally defined brain and peripheral cells of critical relevance to health and disease. Moreover, the three-in-one approach (detecting three types of RNAs: TNEs, mRNAs, and ncRNAs) offers simplicity and noise reduction compared to approaches relying on separate methodologies, experiments, and source materials for assaying enhancers and mRNAs. LcrRNAseq offers advantages to RNA sequencing of brain region homogenates (a suspension of all types of glial, neuronal, immune, and vascular cells resident in a tissue block) or of sorted nuclei without precise information on their three-dimensional origins in human brain and morphological features44,45. Conversely, fluorescent in situ sequencing (FISSEQ) and other in situ hybridization-based methods preserve valuable positional information, but the number of transcripts probed has been limited46.

This analysis showed that putative enhancers active in dopamine neurons link genetic variation to neuropsychiatric traits. It has clear applications for the genetics of more than 20 million patients in the United States alone with perturbed dopamine systems, in narrowing the search window for functional associations and therapeutic nodes, and for defining the regulatory networks that underpin this archetype of a human brain neuron.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41593-018-0223-0.

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**Author contributions**

X.D. performed data analysis with important contributions from D.G., B.G., G.L., C.B., and T.W. T.G.B., C.H.A., M.P.F., P.T.N., J.C.H., R.L.M.F., and C.R.S. obtained and clinically and neuropathologically characterized patient samples. Z.L. and D.G. were responsible for laser-capture and RNA-seq data production. Z.L. and Y.B. performed validation experiments. F.M. and Y.H. designed and performed zebrafish experiments. C.B., P.R., and P.H. performed CAGE experiments. C.R.S. and X.D. wrote the paper with input from all other authors. C.R.S., J.S.M., F.M., A.A.C., and J.J.L. oversaw data analysis and interpretation. C.R.S. conceived, designed, analyzed, and interpreted the study.

**Competing interests**

C.R.S. has collaborated with Pfizer and Sanofi; has consulted for Sanofi; has served as Advisor to the Michael J. Fox Foundation, NIH, and Department of Defense; is on the Scientific Advisory Board of the American Parkinson Disease Association; received funding from the NIH, the US Department of Defense, the Michael J. Fox Foundation, and the American Parkinson Disease Association; and is named as co-inventor on two US patent applications on biomarkers for PD held in part by Brigham & Women’s Hospital. B.G. is the founder of Pacific Analytics PTY LTD, Australia and is a founding member of the International Cerebral Palsy Genetics Consortium, a member of the Australian Genomics Health Alliance, and is on the Scientific Advisory Board of Iggy Get Out!, Australia. T.G.B. provides consultancies to Prothera and GSK; is on the Advisory Board of Vivid Genomics; and has contracted research with Avid Radiopharmaceuticals, Navidea Biopharmaceuticals, and Aponia Therapeutics. The other authors declare no competing financial interests.

**Additional information**

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Methods

Sample collection and processing. We started with 107 high-quality frozen postmortem human control brain samples identified from Banner Sun Health Institute, Brain Tissue Center at Massachusetts General Hospital, Harvard Brain Tissue Resource Center at McLean Hospital, University of Kentucky, University of Maryland Brain and Tissue Bank, Pacific Northwest Dementia and Aging Neuropathology Group (PANDA) at University of Washington Medicine Center, and Neurological Foundation of New Zealand Human Brain Bank. Detailed quality measures and demographic characteristics of these samples are shown in Supplementary Table 1. Median RNA integrity numbers (RIN) were 7.8, 7.8, and 7.2 for substantia nigra samples (used to laser-capture dopamine neurons), temporal cortex (used to laser-capture temporal cortex pyramidal neurons), and motor cortex samples (used to laser-capture motor cortex pyramidal neurons), indicating high RNA quality. Median postmortem intervals were exceptionally short, with 3 h for substantia nigra, 3 h for temporal cortex, and 13 h for motor cortex samples, further consistent with highest sample quality (Supplementary Table 1).

The 107 brain samples represented 93 subjects without clinicopathological diagnosis of a neurodegenerative disease meeting the following stringent inclusion and exclusion criteria. Inclusion criteria: (i) absence of clinical or neuropathological diagnosis of a neurodegenerative disease, for example, PD, according to the UKPDBB criteria9, Alzheimer’s disease according to NIA-Reagan criteria10, or dementia with Lewy bodies by revised consensus criteria11; for the purpose of this analysis incidental Lewy body cases (not meeting clinicopathological diagnostic criteria for PD or other neurodegenerative diseases) were accepted for inclusion; (ii) PMI methods (Applied Biosystems), yielding approximately 7–8 ng RNA per subject. RNA was isolated and treated with DNase (Qiagen) using the Arcturus Picopure method (Applied Biosystems). For each temporal cortex (middle gyrus) or motor cortex sample, about 300 pyramidal neurons were outlined in layers V/VI by their characteristic size, shape, and location in HistoGene-stained frozen sections and laser-captured using the Arcturus Veritas Microdissection System (Applied Biosystems). Total RNA was isolated and treated with DNase (Qiqten) using the Arcturus Picupore method (Applied Biosystems), yielding approximately 7–8 ng RNA per subject. Total RNA was linearly amplified into 5–10 μg of double-stranded cDNA using the validated, precise, isoform-specific RNA amplification method implemented in the Ovation RNA-seq System V2 (NuGen)10. Unlike PCR-based methods that exponentially replicate original transcript and copies, with this method only the original transcripts are linearly replicated25, and amplification is initiated at the 3' end as well as random, thus allowing for amplification of both mRNA and nonpolyadenylated transcripts10. Sequencing libraries were generated from 500 ng of the double-stranded (ds) cDNA using the TruSeq RNA Library Prep Kit v2 (Illuna), according to the manufacturer’s protocol. The cDNA was fragmented, and end repair, A-tailing, adaptor ligation were performed for library construction. Sequencing library quality and quantity control was performed using the Agilent DNA High Sensitivity Chip and qPCR quantification, respectively. Libraries were sequenced using 50 Cycle kits (paired-end) on Illumina HiSequ 2500 and 2500 at the Harvard Partners Core.

Genotyping and imputation. Each sample was genotyped using the Infinium Omni2.5Exome-8 BeadChips (Illumina), which includes more than 2.5 million polymorphic SNPs. The total 98 samples from 93 subjects were genotyped in three batches, with technical replicates for five subjects. We computed pairwise differences between replicates using PLINK2, and reached an average proportion of 0.9991 IBD. Thus, we kept unique sample and replicates in batch 1 for further quality control analysis. We applied PLINK2e (v1.9beta) and in-house scripts to perform rigorous subject and SNP quality control (QC; Supplementary Fig. 1a) that included (i) SNP GC score filtering, (ii) subject call rates, (iii) gender misidentification, (iv) genotype call rates, (v) Hardy–Weinberg equilibrium testing, (vi) test mises (i) heterozygosity outliers, and (vii) IBS/IBD filtering. In total, we excluded 5,249 SNPs with GC < 0.25; 1,955 SNPs not in the genome assembly we used (hg19); 20,049 SNPs with call rates < 95%; 57 SNPs with Hardy–Weinberg equilibrium P < 10−6; 1,295,546 SNPs with MAP < 0.05; and two subjects with IBS/IBD PI_HAT > 0.9. In total, 91 subjects with 1,235,673 SNPs passed QC. We employed SHAPEIT2e (v2.5) to perform pre-phasing and then IMPUTE2e (v2.3.1) to impute the post-QC genotyped markers in autosomal chromosomes using reference haplotype panels from the 1000 Genomes Project (Phase 3), which include a total of 77.8 million SNPs in 2,504 individual samples. For genotyped markers in chromosome X, we used the 1000 Genomes Project Phase I Integrated Release Version 3 as reference haplotype in 1,992 individuals. The genotyped calls of imputed genotypes with posterior probability 0.9 were marked as missing, and we kept biallelic genotypes for further analysis. After genotype imputation, we filtered out imputed SNPs with MAF < 0.05 and info metric < 0.5 that had been compared in a previous review12, which resulted in 4,889,047 imputed SNPs. In total, 6,124,720 SNPs were passed to downstream eQTL analysis.

RNA sequencing data analysis pipeline. RNA-seq raw files in FASTQ format were processed in a customized pipeline. For each sample, we first filtered out reads that failed vendor check or were too short (<15 nt), after removing the low-quality ends and possible adaptor contamination using fastq-mcf with options “-c 5:30:15 -a 1”, “-c 5:20:15 -a 1”, “-c 0:15 -a 1”, respectively. We then checked the q20, q30 and generated k-mer profile using kpal23 for the remaining reads. Reads were then mapped to the human genome (GRCh37/hg19) using TopHat2 (v2.0.8) by allowing up to two mismatches and 10 multiple hits. Reads mapped to ribosomal RNAs or to the mitochondrial genome were excluded from downstream analysis. Gene expression levels were quantified using FPKM (fragments per kilobase of transcript per million mapped reads). Only uniquely mapped reads were used to estimate FPKM. To calculate normalized FPKM, we first ran Cuffquant (v2.2.1) with default arguments for genes annotated in GENCODE (v19), and then ran Cuffnorm with parameters “–total-hits-norm–library-norm-method quartile” on the CBX files generated from Cuffquant.

Sample QC based on RNA-seq data. We performed sample QC similarly to ‘1 Toen PA et al.14. In brief, we ran k-mer profiling for filtered reads using kpal23, and calculated the median profile distance for each sample. Samples with distances differing more than 50% from the rest of the samples were marked as outliers (Supplementary Fig. 1c). We also calculated pair-wise Spearman correlations of gene expression quantification across samples and measured the median correlation (D-statistics) for each sample (Supplementary Fig. 1b,d). Samples with D-statistics markedly different from the rest of the samples were deemed outliers. Moreover, we tested for concordance between reported clinical sex and sex indicated by the expression of female-specific JOST gene and male-specific Y-chromosome gene (Supplementary Fig. 1e). Samples from the first batch with a relative low sequencing depth were also excluded. In addition to these samples used for cell-type-specific transcriptome analyses, we analyzed various additional control samples (for example, amplification controls, tissue homogenate) and technical replicates (Supplementary Fig. 1f–h). In the end, 106 of 115 samples passed QC and were used for downstream analysis (Supplementary Fig. 1a,e).

Defining the cumulative transcribed region by RNA-seq. Previously, ENCODE reported that, in cell lines, 62.1% (cumulatively) of the genome was transcribed with at least five mapped reads (Supplementary Table 11 of ref.13). In our study, we rigorously accounted for sequencing depth and thus considered a genomic sequence as transcribed only if it had a read coverage of more than 0.05 RPM (unique reads per million). This corresponds to approximately 10 mapped reads (considering that for each sample we had, on average, 178 merged reads). With this rigorous definition, we showed that the cumulative coverage of transcribed regions in the dopamine neuron samples is 64.4%.

Defining catalogs of expressed ncRNAs and mRNAs. Normalized expression values of the 106 samples that passed QC were used as input. We first excluded genes with FPKM of zero in all 106 samples. Next, surrogate variable analysis and batch adjustment was performed using the sva4 and ComBat65 packages in R. In brief, the FPKM values were log10 transformed after adding a pseudocount of 0.0001. FPKM values within each group were adjusted for age, sex, and RIN, as well as hidden covariates, using surrogate variable analysis (sva). ComBat was used to adjust for batch effects. Median expression values for each gene were calculated for each cell type. To rigorously exclude low-abundance genes, genes with median adjusted FPKM < 0.01 in a cell type were not considered expressed in that cell type. ENCODE genes meeting these criteria were used to create a detailed catalog of mRNAs and ncRNAs expressed in a cell type.

Genes ‘exclusive’ to dopamine neurons, pyramidal neurons, or non-neuronal cells, respectively, were defined as those that achieved a median FPKM ≥ 0.01 in only one of these three cell types (with adjusted FPKM < 0.01 in each of the other two cell types). We used the t-SNE package in R for t-distributed stochastic neighbor embedding and the heatmap package in R for clustering and visualization purposes of cell-type-exclusive ncRNAs and mRNAs.

Definition of TNE regions. A schematic of the TNE identification pipeline is shown in Fig. 1d and a flow chart in Supplementary Fig. 14a. TNE identification and visualization purposes of cell-type-exclusive ncRNAs and mRNAs.

Definition of TNE regions. A schematic of the TNE identification pipeline is shown in Fig. 1d and a flow chart in Supplementary Fig. 14a. TNE identification analysis was performed separately for dopamine neurons, pyramidal neurons, and non-neuronal cells. We first calculated reads density values (in RPMs) at each genomic nucleotide position for all samples. We then calculated the aggregation score for each cell type by computing the trimmed mean (for example, trimming the 10% highest and lowest data points) of RPMs across the total n samples.

RC
from the cell type of interest for each nucleotide position. We then scanned this aggregation signal in UCSC BegGraph format with a six-step filter:

1. Scan each nucleotide position to filter for (keep for analysis) genomic regions with RPMs higher than the background level. The background level is defined as the average read density across the nuclear genome (i.e., the sum of all RPMs in a cell type divided by the total number of base pairs comprising the nuclear genome, for example, 3,095,677,412 for hg19). The borders of the selected genomic regions for each candidate defined by the first and the last nucleotide for each TNE site that met the RPM cutoff;

2. For each candidate region from step 1, require the summit RPM (i.e., maximal RPM in the region) to achieve a detection \( P \leq 0.05 \) compared to transcriptional background noise. The transcriptional background was defined by randomly selecting 1,000,000 single nucleotide positions outside of the EXCLUSION region (see Methods) and calculating the distribution of their RPMs. The background signal was fitted to a normal distribution using the fitdist(x,norm) function in R. See Supplementary Fig. 14b for the distribution of background signals. Neighboring regions were merged into one region if the genomic distance between them was less than 100 bp;

3. Exclude any regions overlapping with the EXCLUSION regions defined below (for example, known genes, CAGE-defined promoters, and genomic gap regions);

4. Require candidate regions to be longer than 100 bp;

5. Exclude candidate regions containing junction sites supported by more than ten spliced reads in each of at least five samples. Junction sites were defined by randomly selecting 1,000,000 single nucleotide positions outside of the EXCLUSION regions) with length distributions matched to each TNE set. The \( P \) threshold of 0.8. Nonassociated SNPs were extracted using SNAP\(^\text{TM}\) from either of three populations in the 1000 Genomes Pilot 1 dataset with distance limit of 250 kb and linkage disequilibrium (LD) threshold of 0.8. Nonassociated SNPs were extracted using dbSNP (build mm10) to calculate the associated and nonassociated SNPs that physically localized (or did not localize) to TNEs, promoters (unique locations of all GENCODE v19 protein-coding gene TSSs \( \pm 200 \) bp), exons (unique locations of all GENCODE v19 protein-coding transcript inner exons), or random regions (100,000 genomic regions of 400 bp randomly selected beyond the TNEs, FANTOM5 permisive enhancers, and EXCLUSION regions defined above), respectively. Only diseases/trait associated with more than three associated SNPs localizing to TNEs were considered for this analysis. For each genomic feature associated with a disease/trait with an odds ratio greater than one, we performed a Fisher’s exact test. \( P \) values equal to or below 9.64 \( \times 10^{-10} \) (i.e., 0.01 divided by 1,037, the total number of diseases/trait tested in NHGRI-EBI GWAS catalog as of 4 November, 2015) were considered statistically significant.

Validating enhancer activity in HeLa S3 and neuroblastoma cells. PCR primers for the amplification of TNE-defined enhancer candidates and control regions from genomic DNA were designed using the Primer3 web tool and synthesized by IDT Fisher Scientific. All primers sequences are listed in Supplementary Table 6.

The modified vector pGL4.10_pmd3_EF1\(\text{A}\) was kindly provided by RIKEN, and its structure is also described in Supplementary Fig. 9d in their publication\(^\text{13}\). In brief, an EF1\(\alpha\) basal promoter fragment was inserted into HindIII and NheI sites of the promoterless pGL4.10 (Promega) to construct the pGL4.10EF1\(\alpha\) vector, and then the Basal-M and -Sall-containing fragment (as the enhancer insertion site) was removed and reinserted at the SpeI site located upstream of the synthetic poly(A) signal/transcriptional pause site to generate modified versions of the pGL4.10EF1\(\alpha\) vector. The poly(A) site was inserted between the enhancers insertion site and the basal promoter to avoid read-through from the enhancer, since we expect that many of our test elements are transcribed.

The PCR reaction was performed in 50 ml reaction buffer to amplify each sequence of interest from 100 ng of human cerebellum tissue gDNA using a One Taq DNA polymerase Kit (New England Biolabs). The PCR product was digested with BamHI and Sall (New England Biolabs), and the restriction DNA fragment (insert) was isolated using agarose gel electrophoresis and purified by the MinElute Gel Extraction Kit (Qiagen). The pGL4.10_pmd3_EF1\(\alpha\) vector was also digested with BamHI and Sall, and the double-digested DNA (vector) was isolated and purified in the same way as the insert. Using T4 DNA Ligase (New England Biolabs), 100 ng of insert and 20 ng of vector were ligated in 10 ml reaction buffer; 1 ml reaction buffer was transferred to 100 ml of DH5\(\alpha\) competent cells (Invitrogen). Positive colonies were selected by colony PCR and correct insertion in the plasmid was confirmed by sequencing. Cloned plasmids for transfections were purified using the QIAamp DNA Midi Kit (Qiagen).

HeLa-S3 cells were cultured in MEM (Gibco) supplemented with 10% FBS (Gibco) and 100 U/mL penicillin and streptomycin (Gibco). SK-N-MC neuroblastoma cells were cultured in DMEM (Gibco) supplemented with 10% FBS (Nichirei Bioscience Inc.), MEM (WAKO) supplemented with 10% FBS (Gibco), and 100 U/mL penicillin and streptomycin (Gibco). A total of 7.5 \( \times 10^7 \) cells per well

GWSN enrichment analysis. We first downloaded the GWAS-associated SNPs from the NHGRI-EBI GWAS catalog\(^\text{2}\) (v1.0, downloaded on 4 November, 2015), which includes 19,188 human disease/trait association records. For each genomic feature associated with a disease/trait with an odds ratio greater than one, we performed a Fisher’s exact test. \( P \) values equal to or below 9.64 \( \times 10^{-10} \) (i.e., 0.01 divided by 1,037, the total number of diseases/trait tested in NHGRI-EBI GWAS catalog as of 4 November, 2015) were considered statistically significant.
Valuating enhancer activity in zebrafish. Selected TNEs with potential enhancer activity and one negative control element (a nonconserved intergenic sequence region with very low or no signal for enhancer marks such as DNase I hypersensitivity, H3K4me1, or H3K27ac) were amplified from human genomic DNA using primers (Supplementary Table 6). PCR products were purified using Nucleospin Gel and a PCR Clean-up Kit (Macherey-Nagel) and cloned upstream of the zebrafish gata2a promoter 1 to an md2 reporter gene into a modified pdB8996 vector (a gift from D. Balciancs, Temple University). The cloning procedures were performed using an In-Fusion HD Cloning Kit (Clontech) according to the manufacturer’s instructions, into a BamHI linearized vector. Nocodazole was used during a Qagen-tip 20 miniprep kit (Qagen) and verified by restriction digest and sequencing.

Zebrafish stocks (Danio rerio) were kept and used according to Home Office regulations (UK) at the University of Birmingham. For these experiments, the enhancer trap transgenic line Tgemat2:GFP was used. Adults were crossed pairwise and eggs were collected and injected within 20 min after fertilization. Microinjection solution was obtained 20 ng/µl of plasmid DNA and 0.1% of phenol red (Sigma). Injections were performed through the chorion and into the cytoplasm of zygotes using an analog microinjector MINI-1 (Tritech Research). About 150–200 eggs were injected per construct, and experiments were replicated at least three times. Embryos were kept in E3 medium containing 50 µg/ml gentamicin (Thermo Fisher Scientific) and 0.003% phenylthiourea (Sigma) at 28.5°C.

Injected embryos were scored for expression during the first 5 d postfertilization and group images were taken on Zeiss Axios Zoom V16 stereo microscope. Embryos showing specific expression pattern were imaged at the relevant developmental stage on a Zeiss LightBlue and with 20x objective and 0.5 optical zoom. Stacks containing 250–300 slices with 2-µm thickness were acquired, and maximum intensity projections were made using Zeiss ZEN Black Software.

eQTL analysis pipeline. The eQTL analysis was performed for both GENCODE genes and TNEs using the 84 subjects for which lcrRNAseq data from dopamine neurons as well as genotyping data were available. For genes, we first filtered for genes with FPKM > 0.05 in at least ten individuals, then transformed FPKM to rank-normalized gene expression. In brief, the FPKM values were log10 transformed (adding a pseudocount of 0.01). The measurements for each gene were transformed into a normal distribution while preserving relative rankings (quantile normalization) and the mean and sd of the original measurement. For TNEs, the expression distribution was close to a normal distribution and thus quantile normalization was not indicated. Moreover, our TNE identification method already selected for TNEs pervasively expressed across multiple individuals. We then performed surrogate variable analysis (SVA) with the sva R package23 to adjust for potential batch effects in the smaller data set. The selected variables, including batch, age, gender, RIN, PML, and read-length. Adjusted expression values extracted from fva() function were used for downstream eQTL analysis. We used RLE (relative log expression) plots to visually inspect the effects of covariate adjustment. We also filtered out SNPs with missing values or with MAF ≤ 0.05 in the 84 subjects. Matrix eQTL was applied for cis-eQTL analysis, with the cis window defined as 1 megabase between the SNP and the nearest end of a gene or TNE. Nominal P values were generated for SNP–gene pairs in linear regression mode. See Supplementary Fig. 1b for detail.

TNE–host gene function enrichment analysis. We found that 151 cis-regulated TNEs physically localized to intervals of 102 host genes. Gene-set enrichment analysis (GSEA) was performed using the C5 gene sets (GO terms) implemented in the MSigDB database using the hypergeometric test. Each gene set contained genes annotated to the same GO term. For each gene set, the hypergeometric test was performed for k = 1, K, N – k, and n, where k is the number of TNE host genes that are part of a GO term gene set, K is the total number of genes annotated to the same GO term gene set, N is the total number of all known human genes, and n is the number of genes in the query set. The top 50 GO terms enriched in these TNE host genes are shown in Supplementary Table 8 (all with FDR q < 0.05).

We also evaluated whether there was specific enrichment among cis-regulated TNEs in genes associated with brain disorders. We used diseases in MeSH C10 (nervous system diseases) or F03 (mental disorders) for brain disorders, TNEs in genes associated with brain disorders. We used diseases in MeSH q is the total number of genes in the query set. The top 50 GO terms enriched in these TNE host genes are shown in Supplementary Table 8 (all with FDR q < 0.05).

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and RPL13, was used to control for RNA loading. Control samples lacking template and those lacking reverse transcriptase showed virtually no detectable expression. Relative expression abundance of each of the target genes was compared in subjects carrying one or two risk alleles (CT or TT) and those without risk allele (CC) at rs17649553. A two-tailed Student’s homoscedastic t test was used to determine statistical significance. Data are visualized in Supplementary Fig. 12.

Technical confirmation of lcrRNAseq eQTL results in laser-captured dopamine neurons by qPCR. We confirmed the lcrRNAseq-based dopamine neuron eQTLs for KANSL1-TNE1 and LRRG37A4P, respectively, using SYBR Green qPCR (Life Technologies). The geometric mean of two reference genes, EIF4A2 and RPL13, was used to control for RNA loading. For this confirmatory experiment, laser-captured dopamine neuron samples from 35 substantia nigra samples (also used for lcrRNAseq) were analyzed. Data are visualized in Supplementary Fig. 12.

Postmortem brain CAGE methods. Four human postmortem brains (healthy controls) were obtained from University of Maryland, University of Washington, and McLean Hospital, with the same inclusion/exclusion criteria as described above. Substantia nigra tissue samples were used for cap analysis gene expression (CAGE). We extracted $5 \mu g$ of total RNA from each sample using the RNeasy RNA Kit (Qiagen) with RNA integrity number (RIN) > 6. Use of postmortem samples for expression analysis was approved by the IRB of Brigham & Women's Hospital. Libraries were constructed using a published CAGEseq protocol adapted for next-generation sequencing7. Briefly, cDNA was synthesized from total RNA using random primers, and this process was carried out at high temperature in the presence of trehalose and sorbitol to extend cDNA synthesis through GC-rich regions in 5’t untranslated regions. The 5’ ends of messenger RNA within RNA-DNA hybrids were selected by the cap-trapper method and ligated to a linker so that an EcoP15I recognition site was placed adjacent to the start of the cDNA, corresponding to the 5’ end of the original mRNA. This linker was used to prime subsequent strand cDNA synthesis. Subsequent EcoP15I digestion released the 27-base pair (bp) CAGEseq reads. After ligation of a second linker, CAGEseq tags were polymerase-chain reaction amplified, purified, and sequenced on the HiSeq 2000 (Illumina) using standard protocol for 50-bp single-end runs.

CAGEseq data were filtered for CAGEseq artifacts using TagDust79 (version 1.1.2), removal of reads mapping to known ribosomal RNA genes and low-quality reads, mapping to the human genome (hg19) using Burrows–Wheelier Aligner (version 0.5.9) for short reads. Reads mapping to autosomes were used to minimize gender and normalization biases for subsequent analysis. Normalization was done based on the amount of reads per million sequence reads.

Data collection, statistical analysis, and data presentation. Sample sizes were based on the total number of available high-quality brain samples that met inclusion and exclusion criteria. No statistical methods were used to predetermine sample sizes, but our sample sizes are consistent with those recommended by the Genotype-Tissue Expression Consortium80. No randomization of data collection, statistical analysis, and data presentation. gender and normalization biases for subsequent analysis. Normalization was done based on the amount of reads per million sequence reads.

Data distribution was assumed to be normal, but this was not formally tested, and those lacking reverse transcriptase showed virtually no detectable expression. Relative expression abundance of each of the target genes was compared in subjects carrying one or two risk alleles (CT or TT) and those without risk allele (CC) at rs17649553. A two-tailed Student’s homoscedastic t test was used to determine statistical significance. Data are visualized in Supplementary Fig. 12.

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- 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)

Software and code

Policy information about availability of computer code

Data collection

All code for the data collection is available at https://github.com/sterding/BRAINcode.

Data analysis

- PLINK2 (v1.9beta), SHAPEIT2 (v2.5), IMPUTE2 (v2.3.1), fastq-mcf, FastQC, kpal, Tophat(v2.0.8), Cuffquant (v2.2.1), sva, ComBat, UCSC Kent Utilities, eulerAPE, Primer3web (v4.0.0), Marix-eQTL, TagDust (v1.12), BWA (v0.5.9), R (v3.4.4)

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BRAINcode RNA-seq and genotyping raw data have been deposited in dbGAP under accession number phs001556.v1.p1. The processed data and eQTL results for
The BRAINcode project can be queried at http://www.humanbraincode.org through a user-friendly interface. The BRAINcode data sets submitted to dbGAP include the raw data used for figures: Fig. 1b-e, Fig. 2a,c,d; Fig. 3a,3c-d; Fig. 4a; and Fig. 5a-c,5e-g.

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Life sciences study design

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

**Sample size**
Sample sizes were based on the total number of available high-quality brain samples that met inclusion and exclusion criteria. No statistical methods were used to pre-determine sample sizes but our sample sizes are consistent with those recommended by the Genotype-Tissue Expression Consortium (GTEX consortium, Nature, 2017).

**Data exclusions**
Inclusion criteria: (1) absence of clinical or neuropathological diagnosis of a neurodegenerative disease e.g. Parkinson’s disease according to the UKPDBB criteria45, Alzheimer’s disease according to NIA-Reagan criteria, dementia with Lewy bodies by revised consensus criteria. For the purpose of this analysis incidental Lewy body cases (not meeting clinico-pathological diagnostic criteria for PD or other neurodegenerative disease) were accepted for inclusion. (2) PMI ≤ 48 hours; (3) RIN48 ≥ 6.0 by Agilent Bioanalyzer (good RNA integrity); (4) visible ribosomal peaks on the electropherogram. Exclusion criteria were: (1) a primary intracerebral event as the cause of death; (2) brain tumor (except incidental meningiomas); (3) systemic disorders likely to cause chronic brain damage. We also included eight non-brain tissue samples as controls, including five samples of peripheral blood mononuclear cell (PBMC) and three fibroblasts (FB), provided by Harvard Biomarker Study and Coriell Institute. This study was approved by the Institutional Review Board of Brigham and Women’s Hospital.

**Replication**
Attempts at replication were successful. Replication of TNE was performed in four independent cohorts as delineated in Fig. 3. Moreover, select TNE were confirmed by a second method, qPCR, as shown in Fig. 3.

The inverse eQTL relation between the lead GWAS-derived SNP rs17649553 and KANSL1-TNE1 and LRRC37A4P, respectively, was confirmed by a second method, cell type-specific qPCR (Supplementary Fig. 12a). Moreover, this association was independently replicated in a second cohort of neurons laser-captured from 31 high-quality control brains (Supplementary Fig. 12b, Supplementary Table 12). Furthermore, the rs17649553-LRRC37A4P eQTL association was further confirmed in 56 substantial nigra and 96 frontal cortex samples from GTEx (Supplementary Fig. 12c,d), which used a polyA+ selecting protocol that does not allow for assaying KANSL1-TNE1 RNA.

**Randomization**
Allocation was not random and covariates (such as age, sex, PMI) were adjusted in the analysis.

**Blinding**
All samples were from controls (see eligibility criteria above). Blinding to case/control status is not applicable.

Reporting for specific materials, systems and methods

**Materials & experimental systems**

| n/a | Involved in the study |
|-----|-----------------------|
| ✗   | 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 |

**Eukaryotic cell lines**

Policy information about cell lines

**Cell line source(s)**
HeLa and SK-N-MC cell lines were obtained from ATCC.

**Authentication**
HeLa and SK-N-MC cells were used from ATCC and their identity was confirmed by microsatellite testing.

**Mycoplasma contamination**
All cell lines tested are negative for mycoplasma contamination.
SK-N-MC cells were used from ATCC and their identity was confirmed by microsatellite testing.

**Animals and other organisms**

**Laboratory animals**
Zebrafish (Danio rerio) were used. Both males and females, adults, and embryos were used.

**Wild animals**
The study did not involve wild animals.

**Field-collected samples**
The study did not involve samples collected from the field.

**Human research participants**

**Population characteristics**
Characteristics are shown in Supplemental Table 1. Briefly, the mean age at death (standard deviation) was 81 (10.2) for autopsy brains used for lcrNAseq of nigral dopamine neurons. The male:female ratio was 2:1. The median post-mortem interval (stdev) was 3 hours (6.6 hours). The median (stdev) RIN number was 7.8 (0.8).

**Recruitment**
We started with 107 high-quality, frozen postmortem human control brain samples identified from Banner Sun Health Institute, Brain Tissue Center at Massachusetts General Hospital, Harvard Brain Tissue Resource Center at McLean Hospital, University of Kentucky ADC Tissue Bank, University of Maryland Brain and Tissue Bank, Pacific Northwest Dementia and Aging Neuropathology Group (PANDA) at University of Washington Medicine Center, and Neurological Foundation of New Zealand Human Brain Bank.