Spatiotemporal Regulation of Multiple Overlapping Sense and Novel Natural Antisense Transcripts at the Nrgn and Camk2n1 Gene Loci during Mouse Cerebral Corticogenesis

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Nrgn and Camk2n1 are highly expressed in the brain and play an important role in synaptic long-term potentiation via regulation of Ca2+/calmodulin-dependent protein kinase II. We have shown that the gene loci for these 2 proteins are actively transcribed in the adult cerebral cortex and feature multiple overlapping transcripts in the sense and antisense orientations with alternative polyadenylation. These transcripts were upregulated in the adult compared with embryonic and P1.5 mouse cerebral cortices, and transcripts with different 3’ untranslated region lengths showed differing expression profiles. In situ hybridization (ISH) analysis revealed spatiotemporal regulation of the Nrgn and Camk2n1 sense and natural antisense transcripts (NATs) throughout cerebral corticogenesis. In addition, we also demonstrated that the expression of these transcripts was organ-specific. Both Nrgn and Camk2n1 sense and NATs were also upregulated in differentiating P19 teratocarcinoma cells. RNA fluorescent ISH analysis confirmed the capability of these NATs to form double-stranded RNA aggregates with the sense transcripts in the cytoplasm of cells obtained from the brain. We propose that the differential regulation of multiple sense and novel overlapping NATs at the Nrgn and Camk2n1 loci will increase the diversity of posttranscriptional regulation, resulting in cell- and time-specific regulation of their gene products during cerebral corticogenesis and function.

Keywords: CaMKII, P19 teratocarcinoma cells, serial analysis of gene expression and fluorescent in situ hybridization

Introduction

For many years, natural antisense transcripts (NATs) have been implicated in various gene regulatory mechanisms found in prokaryotes (Kumar and Carmichael 1998; Simons 1988) and eukaryotes (Kumar and Carmichael 1998; Terryn and Rouze 2000; Chen et al. 2004; Katayama et al. 2005; Ge et al. 2006; Nagano et al. 2008; Pandey et al. 2008). It is estimated that 8–20% of well-characterized protein-coding genes have at least one overlapping NAT (Fahey et al. 2002; Shendure and Church 2002; Kiyosawa et al. 2003; Yelin et al. 2003; Chen et al. 2004). Overlapping NATs are usually noncoding, often expressed simultaneously with the sense transcript and are regulated by an independent promoter (Dahary et al. 2005; Gingeras 2007). It has been proposed that NATs are associated with brain development (Korneev et al. 2008; Smalheiser et al. 2008; Tochitani and Hayashizaki 2008) and various human disorders such as breast, renal, and colon cancers (Thrash-Bingham and Tartof 1999; Yamamoto et al. 2002; Berteaux et al. 2008), human follicular lymphoma (Capaccioli et al. 1996), Beckwith-Wiedemann syndrome (Smilinich et al. 1999), and alpha-thalassemia (Tufarelli et al. 2003). Little is known about the functional properties of NATs in the mammalian system. To date, only a few functionally characterized mammalian NATs have been reported, these include, Eef-2 (Feng et al. 2006), Air (Sleutels et al. 2002), HOTAIR (Rinn et al. 2007), and Kcnq1ot1 (Pandey et al. 2008). These transcripts regulate gene expression through transcriptional activation (Eef-2), transcriptional repression (HOTAIR and Kcnq1ot1) or chromatin/chromosome modifications, and silencing (Xist and Air) during development. Mounting evidence for a functional role of NATs in development and disease has created much interest in characterizing noncoding transcripts.

We recently reported novel characteristics of multiple overlapping NATs at the Sox4 and Sox11 gene loci in the developing mouse cerebral cortex using serial analysis of gene expression (SAGE) (Ling et al. 2009). We showed that these overlapping NATs are expressed in multiple variant forms in a spatiotemporal manner during cerebral corticogenesis. Based on the same SAGE data sets, we have identified and now describe in detail 2 similar gene loci that encode for the Nrgn and Camk2n1 proteins in the adult cerebral cortex. The NATs generated from these gene loci complementary to the sense transcripts thus may have the potential to regulate the Nrgn and Camk2n1 sense transcripts. Nrgn and Camk2n1, both play a pivotal role in synaptic long-term potentiation via regulation of Ca2+/calmodulin-dependent protein kinase (CaMK) II (Chang et al. 1998, 2001; Pak et al. 2000; Lisman et al. 2002). Differential regulation of these NATs, may therefore alter the regulatory effects of Nrgn and Camk2n1 during synaptic LTP in long-term memory formation. The
purpose of this study is to validate the existence of the noncoding RNA species produced by the Nrgn and Camk2n1 loci and determines if they do indeed interact with their respective protein-coding messenger RNA (mRNA) or sense transcript.

CaMKII is involved in synaptic LTP signaling, which underlies the molecular basis of learning and memory consolidation (Lisman et al. 2002). CaMKII is highly expressed at synapses and constitutes 1–2% of the total protein in the brain. CaMKII requires activation through binding of Ca\(^{2+}\) and calmodulin (CaM) (Gerendasy and Sutcliffe 1997; Kennedy 1998; Lisman et al. 2002). The activated state of CaMKII is subsequently maintained in a Ca\(^{2+}\)-independent manner through phosphorylation of a threonine residue (Thr286 in the α-isofrom and Thr287 in the other isoforms). CaMKII null and autophosphorylation-negative mice show severe deficits in LTP and spatial learning (Silva et al. 1992; Giese et al. 1998) highlighting the importance of this gene in central nervous system function. Therefore, direct or indirect inhibition of CaMKII activation will alter CaMKII-mediated synaptic LTP.

Nrgn is a protein kinase C substrate and a Ca\(^{2+}\)-sensitive CaM-binding protein. Nrgn is highly expressed in the cell bodies and dendritic processes of neurons within the cerebral cortex, hippocampus, and striatum (Represa et al. 1990; Gerendasy and Sutcliffe 1997; Prichard et al. 1999). Due to its high level of expression and ability to regulate Ca\(^{2+}\) and CaM (Baudier et al. 1991; Huang et al. 1993), Nrgn is a potent regulator of postsynaptic signal transduction pathways (Gerendasy and Sutcliffe 1997; Prichard et al. 1999). This protein also plays an important role in synaptic LTP (Klann et al. 1992; Fedorov et al. 1995) with Nrgn knockout mice showing impaired spatial learning and changes in the induction of hippocampal long as well as short-term plasticity (Pak et al. 2000). Nrgn knockout mice also demonstrates lower basal levels of activated CaMKII as compared with wild-type mice, suggesting that Nrgn is crucial for fine-tuning of the amplification of the Ca\(^{2+}\) signal and subsequently CaMKII activation (Pak et al. 2000).

Camk2n1 is also regulated by 2 endogenous inhibitors known as Camk2n1 and Camk2n2 (Chang et al. 1998, 2001). In the adult rat brain, Camk2n1 is highly expressed in the neocortex and the hippocampus, whereas Camk2n2 is predominantly expressed in the hindbrain. These inhibitors are capable of interacting with the catalytic domain of CamkiK in the presence of CaM thus inhibiting the CaM-dependent and autonomous activity of Camk2n1 in a noncompetitive manner (Chang et al. 1998, 2001). Upregulation of Camk2n1 but not Camk2n2 mRNAs has been demonstrated in the mouse hippocampus and amygdala after background contextual fear conditioning. Consequently, Camk2n1 is proposed to play a physiological role in regulating CaMKII activity during the early stages of memory consolidation (Lepicard et al. 2006).

We describe and confirm the spatiotemporal expression profiles of multiple Nrgn and Camk2n1 sense and novel overlapping NATs in various adult mouse organs and differentiating neuronal/glial cells. We used RNA fluorescent in situ hybridization (FISH) to demonstrate that these overlapping NATs form cytoplasmic double-stranded RNA aggregates with their sense counterparts implying they play a role in post-transcriptional regulation of these mRNAs.

Materials and Methods

Genomic Clustering Analysis of Adult-Specific Cortical Transcripts
We performed a genomic clustering analysis to identify actively transcribed genomic loci in the adult cortex using 3 lists of SAGE tags (a, b, and c) representing differentially upregulated transcripts in the adult compared with embryonic development day (E) 15.5, E17.7, or postnatal development day (P) 1.5. See Supplementary Information 1 (SI-1) for a full list of the 144 adult-specific SAGE tags used. These lists were obtained from a SAGE study on cerebral corticogenesis (Gene Expression Omnibus accession number: GSE15031) (Ling et al. 2009). An additional list with 25 165 SAGE tags (all tags from 12 SAGE libraries with tag counts >2) was also obtained from the same study. In order to assess whether any genomic clustering of SAGE tags from lists a-c exist in the adult cerebral cortex, the following methodology was applied: 1) The 25 165 unique tags were mapped to the mouse genome. 2) Within windows of 10 consecutive tags, the number of adult-specific SAGE tags from each list (a-c) was counted. We used a window size of 10 consecutive tags and evaluated the significance based on the empirical distribution of tag counts in 1000 permutations. A previously described permutation based approach (Gotter et al. 2004) was adapted to evaluate the significance of genomic windows. 3) The probability of chance occurrences of clusters of 2, 3, 4, and 5 adult-specific SAGE tags within a window of 10 adjacent tags present on each chromosomal location, irrespective of genetic distance was calculated. The method was reimplemented using the statistical computing environment of R (http://www.r-project.org). 4) We focused on genomic loci with a significant clustering of differentially expressed tags (DETs) with a P value <0.05.

Breeding and Handling of Animals
All experiments were performed according to protocols approved by the Melbourne Health Animal Ethics Committee (project numbers 2001.045 and 2004.041) and the University of Adelaide Animal Ethics Committee (S-086-2005). All mice involved in the study were C57BL/6 unless otherwise specified. Mice were kept under a 12:12 h light-dark cycle with unlimited access to food and water and were culled by CO\(_2\) inhalation. Procurement of brain tissues and mouse organs was carried out according to the method previously described (Ling et al. 2009).

Total RNA Isolation and First-Strand cDNA Synthesis
Genomic DNA-free total RNA was extracted from harvested organs using TRIzol Reagent (Invitrogen) according to the manufacturer’s protocol. Isolated total RNA was treated with the recombimant DNase I enzyme provided in the DNA-free Kit (Applied Biosystems) according to the manufacturer’s protocol to remove genomic DNA. The concentration and purity of all isolated total RNA were determined using the Nanodrop 1000 spectrophotometer (Thermo Scientific). First-strand cDNA was synthesized using oligo-[dT]\(_{14}\) or strand-specific primers and the SuperScript III Reverse Transcriptase Kit (Invitrogen) according to the manufacturer’s protocol, under an RNase-free condition.

Strand-Specific Reverse Transcribed PCR
For each gene locus, 4 first-strand cDNA synthesis reactions were prepared from total RNA (1000 ng for multiple organ analysis, 400 ng for cerebral cortex analysis, and 200 ng for P19 cell analysis); 1) with forward and reverse primers (GeneWorks), and 200 nM forward and reverse primers (GeneWorks), and 0.02x cDNA template. For qualitative analysis, PCR reactions were set up in a 20 µL final volume consisting of 1x LC480 Probe Master mix (Roche Diagnostic), 200 nM forward and reverse primers (GeneWorks), and 0.02x cDNA template. For quantitative analysis, PCR reactions were set up in a 10 µL final volume with an identical reaction mix plus an
additional 100 nM of Universal ProbeLibrary probe (Roche Diagnostics). The cyclizing parameters were as follows: a predenaturating step at 95 °C for 10 min and 35 cycles (45 cycles for quantitative analysis) of 95 °C (10 s), 60 °C (20 s), and 72 °C (20 s) followed by an additional cooling step at 42 °C (1 min). Both nonquantitative and quantitative PCR steps were carried out using the LightCycler 480 Real-Time PCR System (Roche Diagnostic). See Tables S1 and S2 (SI-2) for a full list of primers used in cDNA synthesis, qualitative, and quantitative PCR.

Relative Quantification Using the Standard Curve Method

The crossing point (Cp) of each amplification signal was calculated based on the second derivative maximum method (Rasmussen 2001). A set of serially diluted cDNAs was used to construct a 4-data point standard curve for every PCR system in each run. A total of 3 reference genes, proteasome (prosome, macropain) subunit, beta type 2 (Psmb2), phosphoglycerate kinase 1 (Pgk1), and hydroxymethylbilane synthase (Hmbs) were used as endogenous controls. The following criteria were adopted in order to define a successful assay; a PCR efficiency between 85% and 115% and an R² > 0.985 in each PCR system/run. Based on a successful standard curve, the amount of starting RNA of both target and reference genes were calculated as a linear function of logarithmic concentration and Cp. All estimated starting amounts were in arbitrary values and were used for intra-sample multiple reference gene normalization. A linear model was fitted to the time course or group of expression values for each gene. Genes differentially expressed between the various stages of development or regions were selected using empirical Bayesian moderated t-statistics, which borrow information between genes (Smyth 2004). Standard errors for the mean expression of target genes at various developmental stages were obtained from the linear model. For each comparison, P values were adjusted to control for a false discovery rate (Benjamini and Hochberg 1995).

Rapid Amplification of cDNA Ends and Southern Analyses

First-strand cDNA synthesis was carried out using equally pooled total RNA extracted from 3 biological replicates of rostral and caudal E15.5, whole E15.5, E17.5, P1.5, and adult (5–6 month old) cerebral cortices. Oligo-dT15 with an adaptor sequence (5′-ACGACGCTCTGCTAG-GACTG-3′) was used to prime the first-strand cDNA synthesis (Ling et al. 2009). Second-strand synthesis or PCR was then carried out using a strand-specific primer and the adaptor primer (Table S3 in SI-2). All specific primers were designed to be complementary to SAGE tags or their upstream sequence. Amplified 3′ rapid amplification of cDNA ends (RACE) products were fractionated by electrophoresis prior to Southern blotting and hybridization of probes. Where possible oligonucleotide probes were designed to be complementary to sequence in between the specific primer-priming site and the tag of interest. Synthetic DNA oligonucleotide probes were 5′ end-labeled using T4 polynucleotide kinase (T4-PNK) (Promega) and [γ-32P]ATP (Amersham) according to the manufacturer’s protocol (Section A.1 and Table S3 in SI-2).

Radioactive RNA In Situ Hybridization

RNA in situ hybridization (ISH) was carried out using paraffin sections of embryonic, postnatal, and adult brains (E15.5, E17.5, P1.5, and P150) and a Nrgn/Camk2n1 sense/antisense [35S]UTP-labeled cRNA probe essentially as described previously (Thomas et al. 2000) (Section A.2 and Table S4 in SI-2).

RNA Fluorescent In Situ Hybridization

RNA probes for fluorescent in situ hybridization (FISH) were labeled using PlatinumBright Nucleic Acid Labeling Kit (Kreatech) according to the manufacturer’s protocol. Two different Universal Linkage System (ULS) haptons/dyes were used for labeling of sense and antisense cRNA probes: Dynomics®+7-ULS (absorbance at 547 nm and emission at 565 nm) for sense cRNA probe and Dynomics®+15-ULS (absorbance at 415 nm and emission at 472 nm) for antisense cRNA probe.

Brain tissues (cerebral cortex, olfactory bulbs, cerebellum, and hippocampus) from P56 mice were dissected in cold phosphate-buffered saline (PBS). Dissected tissues were digested in 0.25% (w/v) of trypsin (Invitrogen) prepared in PBS for 5-10 min at 37 °C with occasional gentle pipetting to homogenize the samples. Cells were pelleted and washed with PBS followed by 30 min fixation in 4% (w/v) paraformaldehyde in PBS at 4 °C, smeared directly onto SuperFrost Plus microscope glass slides (Menzel-Glaser) and dried at 37 °C. The fixed cells were subsequently hydrated through a series of ethanol concentrations into RNase-free water at room temperature. Single-stranded RNA was removed through RNase A treatment (50 μg/mL RNase A in 0.5M NaCl, 10 mM Tris–HCl pH 7.5, and 5 mM ethylenediaminetetraacetic acid) for 10 min at 37 °C followed by ethanol dehydration.

Approximately 80 μL of hybridization buffer (2x sodium chloride sodium citrate [SCC], 2 mg/mL of bovine serum albumin, 10% (w/v) dextran sulfate, and 50% (v/v) formamide) containing 100 μg/mL of herring sperm DNA (Promega) and both labeled sense and antisense cRNA probes to a final concentration of 30 ng/mL each were placed onto each slide. A coverslip was placed over the buffer, and the edges were sealed with rubber cement. Slides with hybridization buffer were denatured at 85 °C for 5 min followed by 16- to 20-h incubation in a humidified chamber (50% (v/v) formamide, 5x SCC) at 55 °C. After hybridization, the coverslips were carefully removed and slides were washed in 2x SSC with 50% (v/v) formamide at 55 °C (3x for 5 min each) followed by 2x SSC at 55 °C (3x for 5 min each), 1x SSC at room temperature for 10 min. Slides were dried at 37 °C, and coverslips were mounted with VECTASHIELD Mounting Medium with 1.5 μg/mL of 4′,6-diamidino-2-phenyldine (DAPI) (Vector Laboratories).

Proliferation and Differentiation of P19 Embryonal Carcinoma Cells

The P19 teratocarcinoma cell line was kindly provided by Dr K. Jensen, School of Molecular and Biomedical Science, University of Adelaide, South Australia, Australia. P19 teratocarcinoma cells were cultured and differentiated into neurons as described previously (Rudnicki and McBurney 1998; Ling et al. 2009). Briefly, P19 cell cultures were maintained in Minimum Essential Medium Alpha (alpha-MEM; Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Invitrogen), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM l-glutamine (hereafter known as P19GM).

For induction of neuronal differentiation, 1 × 106 P19 cells were cultured in suspension form using bacteriological petri dishes. The P19GM medium was used with additional supplementation of 5 × 10−7M all-trans retinoic acid (RA; Sigma). After 4 days of culture in suspension in the presence of RA, P19 cells formed embryoid bodies (EBs). EBs were collected from suspension cultures and replated in adherent culture flasks in the P19GM medium with only 5% (v/v) FCS and without RA supplementation. The cells were allowed to differentiate for 5 days. Total RNA from both the proliferating and differentiating cells was extracted using TRIzol Reagent as described above.

Results

Nrgn and Camk2n1 Genomic Loci Are Over-Represented by DETs

The genomic clustering analysis of SAGE tags revealed 2 genomic loci, encompassing the Nrgn and Camk2n1 genes as being significantly overrepresented by SAGE tags (Table 1). Thirteen SAGE tags mapped to the Nrgn and surrounding genomic locus, 3 of which were mapped to genes located upstream of Nrgn (Table 1; Fig. 1A). Within the Nrgn gene locus, 5 tags were mapped to the protein-coding transcript (sense) and 5 were mapped to the antisense strand. Of these tags, Nrgn_tag4 and Nrgn_tag10 (from list a) and Nrgn_tag11 (from list b) were upregulated in the adult cerebral cortex compared with the embryonic and early postnatal stages of development. Noticeably, Nrgn_tag4 and Nrgn_tag10 (both
Expression of Nrgn and Camk2n1 Sense and NATs during Cerebral Corticogenesis

To confirm the expression of NATs within the Nrgn and Camk2n1 gene loci, we performed strand-specific reverse transcribed-PCR (SS-RT-PCR). Two sets of primers (one at the 5' end and one at the 3' end) were designed complementary to the canonical protein-coding transcripts from each locus. SS-RT-PCR was performed on equally pooled total RNAs from each developmental stage (n = 3 per stage; E15.5, E17.5, P1.5, and P150). All probes showed the expected amplification product (Fig. 2A) in the sense-, antisense-, and oligo-[dT]11-primed RT-PCRs for both Nrgn and Camk2n1. Amplification was only observed in the sense- and oligo-[dT]11-primed RT reactions of the Hmbs control gene, and no amplification products were observed in any of the primer-free reactions confirming the presence of NATs at both the Nrgn and Camk2n1 gene loci. This finding is supported by various FANTOM paired-end ditags sequences (obtained from http://www.ensembl.org/), which were mapped to the antisense strands of Nrgn and Camk2n1 (Fig. 1; Table S5 and S6 in SI-2). Taken together, our results confirm the existence of NATs at both the Nrgn and Camk2n1 gene loci.

We further evaluated the level of expression of the Nrgn and Camk2n1 sense and NATs using strand-specific RT quantitative-PCR (SS-RT-qPCR). SS-RT-qPCR primers were designed near to tag positions, and the SS-RT-qPCR normalized

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Table 1

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<th>Tag name</th>
<th>Sequence</th>
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<th>Chromosomal start position and strand</th>
<th>Tag orientation</th>
<th>Tag counts (per 100,000 tags)</th>
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*The chromosomal position of each tag was based on the NCBI Mouse Assembly Build 37.1.
*The sense transcript orientation is the same direction as the protein-coding transcript, whereas the antisense transcript orientation is complementing the sense.
*These values were used to calculate fold change. The formula (X + 0.5)/(Y + 0.5) was used for the calculation, where X and Y represent the normalized combined tag counts of libraries that were compared.
*The tag which represents the canonical transcript based on the RefSeq annotation.
*DET based on list a-c (SI-1).
*Annotation of these tags was based on short SAGE tags, and the expression profiles were based on the combined tag count of all tags within a cluster (see SI-1 for more information).
expression levels were compared with the corresponding SAGE tag counts. The *Nrgn* sense transcript was upregulated in the adult cerebral cortex with an expression level of ~11 to ~22 times higher than the expression in the E15.5, E17.5, and P1.5 cerebral cortices. Only a slight upregulation of the *Nrgn* antisense transcript was observed in the adult cortex with an expression level of ~2.1 and ~2.4 times higher than the expression at E17.5 and P1.5, respectively (Fig. 2B). Similar to the *Nrgn* sense transcript, the *Camk2n1* sense transcript was also upregulated in the adult cerebral cortex with an
expression level of ~15 to ~19 times higher than the expression at all other developmental stages (Fig. 2C).Remarkably, the Camk2n1 antisense transcript was upregulated in the adult cerebral cortex with expression levels of ~11 to ~20 times higher than the expression observed in the other developmental stages (see Table S7 in SI-2 for full information on the qPCR analysis). SS-RT-qPCR analysis validated the SAGE expression findings for both sense and NATs within the Nrgn and Camk2n1 gene loci and showed upregulation of both Nrgn and Camk2n1 sense and NATs in the adult cerebral cortex as compared with other developmental stages.

**Nrgn and Camk2n1 Sense and NATs Contain Different Polyadenylation Sites**

Although SS-RT-qPCR is capable of discriminating the expression of transcripts transcribed from different strands within a locus, validating an individual SAGE tag using this method is not possible and may be inaccurate due to the overlapping of transcripts in either the sense or antisense orientation. To avoid the overlapping effect of these sense or NATs, we employed strand-specific 3’ RACE and Southern analysis to confirm the expression profile of multiple overlapping transcripts in both sense and antisense orientation in a semi-quantitative manner. 3’ RACE was performed using specific primers that were either complementary to SAGE tag sites or to neighboring 5’ upstream sequence. Specific amplicons generated by 3’ RACE were then identified using independent oligonucleotide probes complementary to sequence in between each SAGE tag and the closest predicted polyadenylation signal (AAUAAA or AUUAAA) or preceding 3’ SAGE tag.

3’ RACE-Southern analysis confirmed the presence of 5 of 10 SAGE tags within the Nrgn gene locus. Two of the confirmed SAGE tags, Nrgn_tag4 and Nrgn_tag10 (originating from the Nrgn sense transcripts), were evidently upregulated in the adult cerebral cortex (Fig. 3Ai,ii). Despite the presence of an imperfect poly(A)n tract downstream of the Nrgn_tag10 (Fig. 3D), the existence of previously reported mRNA sequences such as AK002993 and BC061102 (Fig. 1A), whose transcripts end at the Nrgn_tag10 locus, support the existence of this tag. 3’ RACE-Southern analysis of Nrgn antisense tags confirmed the existence of Nrgn_tag7, Nrgn_tag9, and Nrgn_tag13 (Fig. 3Ai,iii,iv). The analysis also validated the SAGE expression profiles for Nrgn_tag7 and Nrgn_tag13. Nrgn_tag7 was expressed throughout cerebral corticogenesis with obvious expression being observed in the P1.5 and adult cerebral cortices, while Nrgn_tag13 was expressed only in the adult cerebral cortex. Interestingly, Nrgn_tag9 was represented by 2 variants (0.4 and 0.5 kb) and was the only Nrgn SAGE tag strongly expressed in the E15.5 cerebral cortex (0.4 kb) but not other developmental stages. In addition, we observed higher relative expression of the Nrgn_tag7, Nrgn_tag9, and Nrgn_tag10 in the caudal part of the E15.5 cerebral cortex compared with the rostral part. All relative comparisons were carried out based on the strand-specific 3’ RACE-Southern analysis of the Psm2b2 reference gene (Fig. 3C). Strand-specific 3’ RACE-Southern analysis showed that sense and NATs within the Nrgn gene loci have different polyadenylation sites, and these transcripts can be independently regulated in a spatio-temporal manner during cerebral corticogenesis.

**Strand-specific 3’ RACE-Southern analysis on the Camk2n1 gene locus confirmed the existence of 3 of 9 SAGE tags, namely Camk2n1_tag8, Camk2n1_tag9, and Camk2n1_tag10 (Fig. 3B). Camk2n1_tag8 (a sense tag) was expressed in the cerebral cortex throughout all stages of development with higher expression in the adult cerebral cortex as compared with other
Figure 3. Strand-specific 3’ RACE-Southern analysis of Nrgn and Camk2n1 in the developing cerebral cortex. (A) 3’ RACE-Southern analysis of the Nrgn sense (i-iv) and antisense (v-vi) transcripts. (B) 3’ RACE-Southern analysis of the Camk2n1 sense (i) and antisense (ii) transcripts. (C) 3’ RACE-Southern analysis for the housekeeping gene, Psmb2 sense (i) and antisense (ii) transcripts that serve as positive and negative controls, respectively. The names of each SAGE tag and the amplicon size are given in the left panel of each figure. *, DETs, ASP, antisense probe, SP, sense probe. (D) Schematic representation of all 3’ RACE-Southern analysis from (A) and (B). Primers represented in the diagram are referring to the primers used in (A) and (B). Scale bars provide an approximate size ratio for each gene.
developmental stages (Fig. 3B). Although an imperfect poly(A)_n tract was found downstream of the Camk2n1_tag8 locus (Fig. 3D), a previously sequenced AK021100 mRNA (Fig. 1B) supports the existence of this tag. Surprisingly, Camk2n1_tag10 is represented by 4 different variants due to alternative polyadenylation sites. These transcript variants are either expressed up to E17.5 (1.2 kb), only in P1.5 (1.5 kb), or throughout all stages of development with a higher level of expression in the P1.5 and adult cerebral cortices (0.8 and 1.0 kb, respectively). Camk2n1_tag9 represents a transcript from the antisense strand, which was found to be expressed only in the adult cerebral cortex by 3’-RACE-Southern analysis (Fig. 3Bii). We identified 2 polyA signals within the locus where the tag was amplified. Due to the existence of an imperfect poly(A)_n tract downstream of the tag and lack of previously reported sequenced mRNA from the locus, the actual polyadenylation site cannot be confirmed at this stage (Fig. 3D). No obvious differential expression was found for any of the Camk2n1 sense and antisense tags between the rostral and caudal E15.5 cerebral cortices. Similarly to Nrgn, different transcript variants of Camk2n1 were shown to be differentially regulated throughout cerebral corticogenesis.

RNA In Situ Hybridization of Nrgn and Camk2n1 Sense and NATs during Brain Development
To localize the expression of the Nrgn and Camk2n1 sense and NATs in the brain, we performed RNA ISH on coronal and sagittal sections of whole mouse brain from E11.5, E13.5, E15.5, E17.5, P1.5, and P150 (Figs 4–5). In general, the majority of NATs are expressed at low level and therefore radioactively probed Nrgn and Camk2n1 NATs were exposed to liquid emulsion for 3 times (21 days) longer than their counterpart sense transcripts (7 days) (see Section 2.7). ISH of the hemoglobin alpha, adult chain 1 (Hba-a1) using sense/antisense cRNA probes (Fig. S1 in SI-2) were used as controls for all ISH sections. Sections for Hba-a1 ISH were exposed to liquid emulsion for 21 days.

ISH showed that the Nrgn sense transcript was expressed only in the piriform cortex (Fig. 4A) and near the fasiculus retroflexus region (Fig. 4C) at E13.5. However, its expression was gradually extended to the entire cerebral cortex by P1.5 (Fig. 4A). From the sagittal sections, it can be seen that the Nrgn sense transcripts were detectable in the inferior colliculus from E17.5 onwards (Fig. 4C). Strong expression of the Nrgn sense transcript was found in the cerebral cortex, piriform cortex, hippocampus, dentate gyrus, inferior colliculus, and the outer plexiform layer of the olfactory bulb of the adult, with moderate to low expression in the caudoputamen and caudolateral septal nucleus regions (Fig. 4C; Fig. S2 in SI-2). Similar to the Nrgn sense transcript, the Nrgn NAT was also detectable in the developing brain. The Nrgn NAT was first observed in the telencephalon of the E11.5 brain and its expression increased in the cortical plate from E13.5 onwards (Fig. 4B). The NAT expression, however, only became prominent in the cortical plate and outer plexiform layer of the olfactory bulb at E17.5 and P1.5 (Fig. 4D). Under high magnification bright-field microscopy, expression of Nrgn NATs can be observed as exposed silver grains particularly in the developing cerebral cortex. Examples of bright-field micrographs for sense and NATs expression are presented in Figure 4E (see Fig. S3 in SI-2).

ISH showed that the Camk2n1 sense transcript was detected in the mesencephalon and rhombencephalon at E11.5 (Fig. 5A,C). Its expression became prominent at E13.5, in the thalamus, and hippocampal formation. Expression of the Camk2n1 sense transcript was also found in various brain regions including the cortical plate, caudoputamen, hippocampus, dentate gyrus, inferior colliculus, and thalamus between E17.5 and P150 (Fig. 5A,C; Fig. S4 in SI-2). Similar to the Nrgn sense transcripts, the Camk2n1 sense transcript was also expressed in the outer plexiform layer of the adult olfactory bulb (Fig. 5C). In contrast to the sense transcript, the Camk2n1 antisense transcript was only detectable at P150, where weak expression was observed in the cerebral cortex, caudoputamen, and piriform cortex (Fig. 5B,D, Fig. S4 in SI-2). In Figure 5E, high magnification bright-field micrographs show the expression of Camk2n1 NATs as silver grains in the cortical cells of the P1.5 brain. The RNA ISH analysis complements our SS-RT-qPCR and single-stranded 3’ RACE-Southern analyses revealing upregulation of Nrgn and Camk2n1 sense and NATs in the postnatal cerebral cortex. RNA ISH also demonstrated spatiotemporal regulation of these transcripts during cerebral corticogenesis as well as differential and coregulation of Nrgn and Camk2n1 sense and NATs implying that they have a specific role during cerebral corticogenesis.

Expression of Nrgn and Camk2n1 Sense and NATs in Various Mouse Organs
To investigate whether the expression of the Nrgn and Camk2n1 NATs is specific to the brain, we performed SS-RT-qPCR analysis to quantitatively measure the expression level of these transcripts in various regions of the adult mouse brain and other organs. We demonstrated high expression of the Nrgn sense transcript in the adult mouse brain especially in the olfactory bulb, hippocampus, thalamus, and cerebral cortex (Fig. 6A). Its expression was relatively low in the cerebellum, medulla, E15.5 whole brain, and in other adult mouse organs including the heart (cardiac muscle), kidney, liver, skeletal muscle, liver, skin, spleen, stomach (smooth muscle), testis, and thymus. Interestingly, the Nrgn antisense transcript was expressed at a similar level across all organs screened with exception of the skin (where expression was ~4-fold higher than in other mouse organs). In summary, Nrgn sense transcripts are specifically expressed in the brain suggesting they have a crucial role in adult brain function, whereas the NATs are expressed in all organs suggesting novel and fundamental regulatory functions.

The Camk2n1 sense transcript was generally expressed at a higher level in the adult mouse brain with the highest expression observed in the cerebral cortex followed by the hippocampus, thalamus, cerebellum, olfactory bulb, and medulla (Fig. 6B). The Camk2n1 NAT was expressed in a similar pattern as the sense transcript but at a lower level. This finding suggests that both the sense and antisense Camk2n1 transcripts are coregulated across these tissues or organs and are therefore likely to be regulated by similar transcriptional elements (see Tables S8 and S9 in SI-2 for full information on qPCR analysis).

Expression of Nrgn and Camk2n1 Sense and NATs in Proliferating and Differentiating P19 Cells
The high expression of Nrgn and Camk2n1 sense and NATs that we have demonstrated in the postnatal and adult brain
Figure 4. Radioactive RNA ISH analysis of Nrgn in the developing mouse brain. The developmental stage is shown in the upper right corner of each micrograph and the labels to the left of the micrographs correspond to the transcript being assessed. Labels within the micrographs are explained in the lower left corner of the figure. For (A) and (B), scale bars for the E11.5, E13.5, and E15.5 sections = 500 μm, scale bars for the E17.5 and P1.5 sections = 1.0 mm, and the scale bar for the adult sections = 2.0 mm. For (C), the scale bar for the E11.5 sections = 500 μm, scale bars for the E13.5, E15.5, and P1.5 sections = 1.0 mm, the scale bar for the adult sections = 2.0 mm. For (D), the scale bar for the E17.5 and P1.5 sections = 200 μm and the scale bar for the P1.5 section = 500 μm.

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Figure 5. Radioactive RNA ISH analysis of Camk2n1 in the developing mouse brain. The developmental stage is shown in the upper right corner of each micrograph and the labels to the left of the micrographs correspond to the transcripts being assessed. Labels within the micrographs are explained in the lower left corner of the figure. For (A) and (B), scale bars for the E11.5, E13.5, and E15.5 sections = 500 μm, scale bars for the E17.5 and P1.5 sections = 1.0 mm, and the scale bar for the adult sections = 2.0 mm. For (C), the scale bar for the E11.5 sections = 500 μm, the scale bars for the E13.5, E15.5, and P1.5 sections = 1.0 mm, the scale bar for the adult sections = 2.0 mm. For (D), the scale bar for the E17.5 and P1.5 sections = 200 μm and the scale bar for the P1.5 section = 500 μm.
suggest that they have important roles in neuronal and glial cell function. To test this hypothesis, we employed an in vitro model to compare their expression in proliferating and differentiating P19 cells. The P19 cell line was derived from teratocarcinoma cells and can be differentiated into neurons and astrocytes through treatment with RA (McBurney et al. 2001).

Figure 6. SS-RT-qPCR analysis of Nrgn and Camk2n1 transcripts in various adult brain regions, organs, and P19 cells. The vertical bars represent the relative expression (after normalization to 3 housekeeping genes) levels of each sense and antisense transcript for Nrgn (A and C) and Camk2n1 (B and D) in various adult mouse brain regions, organs (A and B), and P19 cells (C and D). n = 3 for all SS-RT-qPCR analysis except for the various brain region analysis where n = 2. Error bars represent the standard error of the mean expression value. **P < 0.01 and ***P < 0.001.
Multiple Overlapping Sense and Novel Natural Antisense Transcripts at the Nrgn and Camk2n1 Gene Loci

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1988; Varga et al. 2008). After 5 days of differentiation with serum deprivation, the majority of P19 cells will differentiate into neurons with glutamate- and GABA-responsive properties (Varga et al. 2008) with the capability to form synapses. We performed 5S-RT-qPCR to evaluate the expression levels of the Nrgn and Camk2n1 sense and NATs in both proliferating and differentiating P19 cells.

The Nrgn sense and NATs were both upregulated in differentiating P19 cells with expression levels of ~9- and ~4-fold higher than in proliferating cells, respectively (Fig. 6C). A more significant increase in expression of the Camk2n1 sense and NATs was observed in the differentiating P19 cells compared with those that were proliferating (~50-fold difference; Fig. 6D; see Table S10 in SI-2 for the qPCR raw data). These observations support our hypothesis that Nrgn and Camk2n1 sense and NATs have an important role in the neuronal/glial cell function.

**Sense and NATs Form Double-Stranded RNA**

Overlapping NATs are 100% complementary to their counterpart sense transcripts. To investigate the interaction between the Nrgn and Camk2n1 sense and NATs and their capability to form double-stranded RNA, we carried out RNA FISH on RNase A treated trypsinized cells that were obtained from the adult cerebrum, cerebellum, olfactory bulbs, and hippocampus. As an RNAse A treatment control, we performed RNA FISH for the Hmbs housekeeping gene on both untreated and RNase A treated cells from the P1.5 olfactory bulbs (Fig. S5 in SI-2). The RNA FISH analysis showed cytoplasmic aggregation of colocalized sense and NATs for both Nrgn and Camk2n1 in all cells studied (Fig. 7). The lowest number of aggregates was observed in cells obtained from the hippocampus for both the Nrgn and Camk2n1 RNA FISH. These observations confirm the ability of the Nrgn and Camk2n1 NATs to form double-stranded RNA products with the sense transcripts indicating that they are involved in posttranscriptional regulation.

**Discussion**

SAGE allows quantification of known and unknown polyadenylated RNA by generating a 3′ 14–17 bp unique tag from each transcript making the approach suitable for comparative transcriptomic studies and discovery of novel transcripts (Scott and Chrust 2001). Using genomic clustering analysis, we have revealed that the Nrgn and Camk2n1 gene loci are represented by multiple SAGE tags in both sense and antisense orientations. A similar clustering analysis approach has been shown to successfully determine gene clusters in *Caenorhabditis elegans* muscle tissue and human medullary epithelial cells, which were proposed to be regulated by cis-acting elements (Roy et al. 2002; Gotter et al. 2004). Our results, however, suggested bidirectional transcription of multiple overlapping sense and novel NATs within these gene loci. These overlapping transcripts have different 3′ untranscribed region (UTR) lengths due to alternative polyadenylation sites and are independently regulated in a spatiotemporal manner during cerebral corticogenesis. These features resemble and further support our previous findings of multiple overlapping sense and NATs at the Sox4 and Sox11 gene loci during embryonic cerebral corticogenesis (Ling et al. 2009).

We report the most comprehensive expression profiling study of multiple Nrgn and Camk2n1 sense and NATs during brain development to date. In general, our analysis complements the findings of previous studies, which have demonstrated prominent expression of the Nrgn and Camk2n1 proteins in adult neurons (Represa et al. 1990; Gerendasy and Sutcliffe 1997; Pritchard et al. 1999; Chang et al. 2001). We have shown that Nrgn and Camk2n1 sense and NATs with different 3′ UTR lengths have different expression profiles during cerebral corticogenesis suggesting meticulous regulation of these transcripts in a cell- and time-specific manner. In addition, Nrgn and Camk2n1 sense and NATs were upregulated in differentiating P19 cells. Neurons generated from P19 cells synthesize acetylcholine and have high affinity uptake sites for GABA defining them as cholinergic and suggesting they have the ability to form synapses (McBurney et al. 1988). Given the properties of the in vitro model used and the Nrgn and Camk2n1 sense and NATs expression profiles, we propose that these NATs may play a pivotal role in neuronal synaptogenesis and plasticity.

A number of features of the 3′ UTR of a transcript control various aspects of the mRNA and its translation into protein. The multiple overlapping sense or NATs with different 3′ UTR lengths identified at the Nrgn and Camk2n1 loci could influence transcript localization within the cellular compartment, transcript stability, and transcript translation rate. Furthermore, 3′ UTRs with various lengths may contain different regulatory elements, allowing fine-tuning of the regulation of protein synthesis or expression (e.g., through modulation of alternative splicing) within specific cell or tissue types (Abdel Wahab et al. 1998; Krebber and Ponstingl 1996; Winter et al. 2007). Different 3′ UTR lengths of transcripts may harbor different target sites for small RNAs such as microRNAs (miRNAs) (Fig. 1; Tables S11 and S12 in SI-2). Transcripts with longer 3′ UTR may be targeted by more miRNAs as compared with transcripts with shorter 3′ UTR, resulting in increased repression of protein translation or mRNA degradation. In addition, AU-rich elements within the 3′ UTR can regulate mRNA stability through deadenylation, decapping, or 3′→5′ decay processes (Wilusz C and Wilusz J 2004). It is likely that one or a combination of these features are involved in the control of the spatiotemporal expression patterns of the Nrgn and Camk2n1 sense and NATs during cerebral corticogenesis.

We have demonstrated the presence of various novel overlapping NATs at the Nrgn and Camk2n1 gene loci in the adult cerebral cortex. There is ample evidence to show that the presence of NATs is relatively common in higher eukaryotes (Fahey et al. 2002; Shendure and Church 2002; Kiyosawa et al. 2003; Yelin et al. 2003; Chen et al. 2004). Overlapping NATs are 100% complementary to their sense counterpart sequences. As we demonstrated by RNA FISH, NATs at the Nrgn and Camk2n1 loci can form double-stranded RNA with the sense transcript and therefore may activate the RNA-induced silencing (RISC) complex-mediated mRNA degradation mechanism or host interferon response. Alternatively, these overlapping NATs could fold into complex secondary structures that serve as templates for the production of both miRNA and/or endogenous small interfering RNA (siRNA) with exceptionally high specificity to the sense transcript. Due to the fact that the Nrgn and Camk2n1 proteins are highly expressed in the mouse and rat brain (Represa et al. 1990; Chang et al. 2001; Lepicard et al. 2006; Saha et al. 2006), respectively, it is not likely that RISC-, miRNA- or endogenous siRNA-mediated regulation of the sense transcripts is
occurring. Since Nrgn and Camk2n1 NATs are localized only in the cytoplasm, it is not likely that these NATs mediate transcriptional activation or silencing of their sense counterparts through interaction at the chromatin level in a similar manner reported for the Air and Kcnq1ot1 (Nagano et al. 2008; Pandey et al. 2008). Based on our observation in the RNA FISH analysis and the current understanding of Nrgn and Camk2n1 protein expression, we propose that these NATs either play

Figure 7. RNA FISH analysis of Nrgn and Camk2n1 sense and NATs. The type of transcripts assessed is shown at the top of the figure, and the origin of the cells are shown to the left of the micrographs.
a role in binding sense RNA preventing protein translation in a reversible manner or serve as templates for endogenous siRNA production, which may then regulate other transcripts.

This study reports for the first time the existence and spatiotemporal regulation of multiple overlapping Nrgn and Camk2n1 sense and NATs variants during cerebral corticogenesis. The differential expression profiles of the Nrgn and Camk2n1 NATs and their capability to form double-stranded RNA with their sense counterpart suggest that they are biologically relevant during brain development particularly cerebral corticogenesis. We propose that multiple overlapping Nrgn and Camk2n1 sense and NATs increase the diversity of postranscriptional regulation, resulting in cell- and time-specific regulation of their gene products.

Supplementary Material
Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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References


