Natural antisense transcripts are co-expressed with sense mRNAs in synaptoneurosomes of adult mouse forebrain

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Abstract

Natural antisense transcripts and overlapping sense transcripts are expressed in a variety of tissues, including adult mouse brain. Here we show that a subset of mRNA-like sense-antisense transcript pairs are co-expressed within synaptoneurosomes of adult mouse forebrain, a subcellular fraction that is enriched in pinched-off dendritic spines of pyramidal neurons. Several of these pairs involve mRNAs that have been implicated in synaptic functions and in Alzheimer disease pathways. This study provides evidence that a new class of noncoding RNAs (natural antisense transcripts) are expressed near synapses, and encourages further studies of their roles in neuronal function.

Keywords

antisense RNA; RNA interference; transcriptome; synaptic plasticity.

1. Introduction

Natural antisense transcripts (NATs) and other long noncoding RNAs (ncRNAs) have emerged as prominent classes within the mammalian transcriptome (Calninci et al., 2005; Kapranov et al., 2007). For example, approximately 2000 pairs of sense-antisense transcripts (SATs) have been identified in mouse (Kiyosawa et al., 2005). SATs are pairs of endogenously encoded transcripts that may be produced from both strands of the DNA and are capable of forming sequences complementary to each other. By using strand-specific oligo DNA chip, Kiyosawa et al. (2005) found that the majority of the SATs are indeed expressed at various levels in variety of tissues. SATs can be derived from both protein-coding genes and non-protein–coding genes, including exonic, intronic, and intergenic regions.

SATs have been identified in various organisms across from humans to yeast, and studies performed in various organisms have suggested that SATs can participate in a broad range of regulatory events (Lapidot and Pilpel, 2006). For example, SAT coexpressed within a cell can lead to various kinds of antisense regulation (Lavorgna et al., 2004), such as RNA masking, RNA editing, and RNA interference (RNAi). In mammals, it is well known that some antisense RNAs are involved in epigenetic regulation of gene expression for the imprinted genes and X-linked genes on the inactive X chromosome. However, biological roles of vast numbers of unannotated SATs are largely unknown, and the functions of NATs and SATs and the mechanisms by which they regulate sense mRNAs, is under active study. Although many of the SATs found in mouse represent atypical transcripts, tend to be localized in nucleus and are not polyadenylated, some natural antisense transcripts are mRNA-like: they bear poly A+ tails and are expressed in the cytoplasm (Cheng et al.,

2005), where they may potentially interact with overlapping sense mRNAs.

Local regulation of mRNA translation in and near dendritic spines appears to be important for synaptic plasticity and cognition (Vanderklish and Edelman, 2005). Many mRNAs have been found to be enriched in synaptic fractions (e.g., Matsumoto et al., 2007) and associated with postsynaptic densities (PSDs) (Suzuki et al., 2007). Natural antisense transcripts were proposed to regulate long-term gene expression of the nervous system, particularly in learning and memory in the mammalian CNS (Smalheiser et al., 2001). Short trans-acting antisense transcripts (i.e., microRNAs) were discovered soon thereafter, and many studies have demonstrated that microRNAs are expressed within the mammalian brain where they play a role in regulating mRNA translation and stability (reviewed in Tai and Schuman, 2006; Kosik, 2007; Fiore et al., 2008). In mouse, dendritic miR-134 represses translation of LIMK1 which regulates dendritic spine growth (Schratt et al., 2006), and in *Drosophila*, mutations in a microRNA pathway component (*armitage*, part of the RNA Induced Silencing Complex, or RISC) block olfactory long-term memory (Ashraf et al., 2006). MicroRNA pathway components including the RNAse III enzyme dicer (that processes microRNA precursors into mature microRNAs), eIF2C (the core RISC component), mature microRNAs, and microRNA precursors are all expressed within dendritic spines and are enriched in association with the postsynaptic density (Lugli et al., 2005, 2008). Besides microRNAs, other noncoding RNAs (notably BC1 in rodents and BC200 in humans) are expressed in dendrites and appear to regulate mRNA translation (e.g., Lin et al., 2008). An increasing number of long noncoding RNAs, including natural antisense RNAs, have been shown to be expressed within adult brain (Mercer et al., 2008).

As described, our group recently reported that many pairs of natural antisense transcripts are

co-expressed with overlapping sense transcripts within a variety of tissues, including adult mouse brain. We also found that small RNAs can be generated only from complementary regions of several SAT pairs (Kiyosawa et al., 2005; Okada et al., 2008). Since SATs in the cytoplasm may serve as a source for siRNA (Watanabe et al., 2008) or other small RNA production (Kapranov et al., 2007), we have asked in the present study whether any of these paired sense-antisense transcripts are co-expressed within synaptic fractions of adult mouse forebrain, particularly among the population of polyadenylated, mRNA-like transcripts supposed to be localized in the cytoplasm.

2. Methods

Synaptoneurosomes (SYN) are a well-characterized synaptic subcellular fraction that is enriched in pinched-off dendritic spines of pyramidal neurons (as well as other adherent material such as closely apposing glial endfeet). These were prepared from 2 month old male C57BL/6 mice, and were previously characterized in terms of their enrichment of synaptic marker proteins and RNAs (Lugli et al.,. 2005, 2008). Each preparation represented a pool of 3-4 animals, and consisted of synaptoneurosomes as well as an aliquot of the total forebrain homogenate (TOT). The preparations studied here are a subset of those examined in Lugli et al, 2008, where it was shown that contamination of synaptoneurosomes with extraneous material is less than ~15%.

Total RNA from two independent preparations was prepared using a modified Trizol method that captures small RNAs well (Lugli et al., 2008) and shipped on dry ice to Tsukuba, Japan. The RNA was rechecked for integrity, 1 microgram was labeled with Cy-dye using oligo dT-priming to selectively label RNAs bearing poly-A sequences, and hybridized to a custom oligo DNA chip as described (Kiyosawa et al., 2005). The first sample preparation was analyzed using a 2-color protocol in which TOT vs SYN samples were compared on the same chips, resulting in paired data points; the second preparation was analyzed using a 1-color protocol in which total and SYN samples were analyzed on different chips. Initial quality control and intensity values for each transcript were processed using Agilent Feature Extraction package. Further data cleansing was carried out to remove outlier spots and spots having high internal pixel variation, and to remove effects due to differences in dye color, chip and sample preparations: First, transcripts were removed if their SYN intensity values exhibited high internal variation (SD > 1/3 the raw intensity value), or

their median SYN intensity values were near background values (value < 2.5 times background). Second, the raw intensity values across the chips were normalized by "mean centering" each of the four groups to the mean intensity value (across the chip as a whole) of one arbitrarily chosen sample (TOT on chip 1). That is, the individual raw intensity values for a given sample on a given chip were divided by the mean of the intensity values across the sample as a whole, and then multiplied by the mean for TOT on chip 1. In this manner, all samples were adjusted to exhibit the same normalized mean value on each chip. It should be noted that all of the results reported in this paper hold whether one considers normalized or non-normalized intensity data.

The annotation of a transcript as “sense” vs. “antisense” is somewhat arbitrary; the original annotation referred to transcripts as “sense” if they arose from the (+) strand of the published mouse genome assembly and “antisense” if they arose from the (-) strand (Kiyosawa et al., 2005. In this study, we simply refer to the transcripts in each pair as "transcript #1" vs. "transcript #2" rather than as "sense" vs. "antisense". Both transcripts in a SAT pair may potentially each have direct functions on their own, and may potentially regulate each other as well.

3. Results

Sense-antisense transcript pairs were said to be co-expressed if each transcript was detected at ≥ 2.5 X background on each of two independent samples run on separate chips. Using that criterion, 121 pairs were co-expressed in the SYN fraction, most of which (95) were detectably co-expressed within the total homogenate as well. (The remaining 26 pairs were reliably detected only in the SYN fraction.) This indicates that the pairs are, indeed, co-expressed, at levels that should permit them to interact with each other and to exert physiological roles within the dendritic compartment. Conversely, only 13 pairs were co-expressed within the total homogenate that were not detected within the SYN fraction (Supplementary File).

Almost all transcripts exhibited SYN enrichment ratios within a 2-fold range relative to the total homogenate (i.e., intensity in SYN/intensity in TOT = 0.5 – 2.0). The highest SYN enrichment (3.29) was observed with a transcript (RIKEN ID ri|8030479B14) having antisense orientation to a ribosomal protein S12 mRNA transcript located on mouse chromosome 7; this is consistent with data that a variety of ribosomal subunit mRNAs are enriched in synaptosomes (Matsumoto et al., 2007). The next highest extent of enrichment (2.11) was observed for beta-amyloid precursor cleaving enzyme (BACE1, see below). In contrast, transcripts that were relatively depleted from SYN include FK506 binding protein 3 (0.42), nucleoporin 62 (Nup62) (0.42), and polymerase (RNA) II (DNA directed) polypeptide C (0.49), which might be expected to have roles predominantly within the nucleus and cell body. Overall, there appeared to be little correlation between the SYN enrichment of a sense transcript and its paired antisense counterpart (not shown), though this may simply reflect the fact that enrichment ratios were confined to a relatively narrow

range. Enrichment ratios of 2-5 are characteristic of proteins and RNAs that are known to play specific roles in or near synapses (e.g., alpha- and beta-dystroglycan, Smalheiser and Collins, 2000; CAMK2a and BC1, Lugli et al., 2008). The enrichment ratios observed for mature microRNAs in the SYN fraction ranged from 0.15 to 4.8 as estimated by microarray (Lugli et al., 2008). The enrichment observed for SATs was particularly modest when compared to core synaptic components such as PSD-95 protein, which shows ~20-fold enrichment within synaptic fractions as estimated by Western blotting (Lugli et al., 2005, 2008). Similarly, we examined mRNA expression in one RNA preparation using an Agilent 44k gene expression profile chip, and found that the highest SYN enrichment was obtained with SAP97, a PSD-95 family member (33.4-fold enriched over total homogenate; data not shown).

Given that the gene chip was designed to provide a broad survey of SATs and did not focus specifically on transcripts expressed within brain, it was interesting to note that several of the transcripts co-expressed as SAT pairs are related to synaptic functions – e.g., SNAP-25 binding protein, catechol O-methyltransferase (COMT), chloride channel 2 (CLC-2), perlecan, and signaling proteins including kinases, phosphatases, and proteasomal components (Supplementary file). Moreover, at least six of the co-expressed pairs involved mRNAs encoding proteins that have been implicated in pathways related to Alzheimer disease (Table 1). These include **BACE1**, the enzyme that performs beta-cleavage of beta-amyloid precursor protein (APP); **reticulon 3**, a protein that binds BACE1 and inhibits APP cleavage; **APP binding protein 2** (Fe65-like) which binds the cytoplasmic tail of APP; **rab6**, a protein that is elevated in Alzheimer cortex; **sirtuin-3**, a member of a class of proteins thought to be neuroprotective in Alzheimer disease; and **integrin-linked kinase**, which phosphorylates GSK3 to inhibit its activity (GSK3 phosphorylates a number of

Alzheimer-related proteins including tau and presenilin-1). A recent paper described a natural antisense transcript for BACE1 (Mercer et al., 2008), but the others have apparently not been described before, even though it has been noted that a variety of Alzheimer-related mRNAs have natural antisense transcripts (e.g., Guo et al., 2006; Parenti et al., 2007). Although the beta-amyloid processing machinery is not thought of as specifically targeted to dendrites, abnormal beta-amyloid processing does affect synaptic components and alters synaptic transmission (Almeida et al., 2005). Transgenic mice overproducing reticulon 3 by only ~2-fold above normal levels produce reticulon-containing neuritic aggregates (Hu et al., 2007). As well, rab6 protein has been localized within dendrites (Pierce et al., 2001). Sirtuin-3 has been genetically linked to human longevity and the sirtuins as a class have been proposed to interact with Alzheimer disease pathways (Anekonda and Reddy, 2006). Thus, these findings extend previous data indicating that sense-antisense regulation may be an important feature of pathways related to Alzheimer disease, and suggest that they may be operative within the dendritic compartment.

Some sense-antisense pairs co-expressed in the SYN fraction were expressed at roughly equal levels whereas others exhibited up to ~40-fold differences between transcript #1 and transcript #2 of the pair (Table 1 and Supplementary file). Note that RAB6 and sirtuin-3 were expressed at roughly equal abundance as their antisense counterparts, whereas BACE1 and reticulon 3 were more abundant and APP binding protein 2 was less abundant than their antisense counterparts (Table 1).

4. Discussion

The present study extends the findings of Kiyosawa et al. (2005), Lugli et al. (2005, 2008) and Mercer et al. (2008). Not only are noncoding antisense transcripts found in neurons in the adult mammalian brain, but a subset of overlapping sense-antisense transcript pairs are co-expressed within synaptoneurosomal preparations, suggesting that they are expressed together within the dendritic and/or dendritic spine compartments of pyramidal neurons. The present data are consistent with the proposal that sense-antisense pairs may be involved in regulation of synaptic plasticity and learning (Smalheiser et al., 2001) and raise the possibility that they may be involved as well in pathways related to Alzheimer disease.

It is not fully clear yet whether mRNA-like sense-antisense pairs hybridize to each other in the cytoplasm *in vivo*, or whether their interactions activate RNA interference/microRNA pathways leading to formation of small RNAs or altered stability or translation of mRNA transcripts. To date, few cloning and sequencing studies have identified endogenous siRNAs within mammalian cells. However, a few recent reports have shown that sense-antisense transcripts can give rise to small RNAs, apparently via RNA interference, in early mouse oocytes, mouse ES cells, zebrafish embryos and the nuclei of Xenopus oocytes (Watanabe et al., 2008; Carlile et al., 2008; Ogawa et al., 2008). Carlile et al. (2008) have suggested that siRNAs may be formed but are generally limited in their expression within cells due to the limited overlap of sense-antisense co-expression and by their transient nature. If so, then adult forebrain may be a favorable place to look for endogenous siRNA formation, since a significant number of sense-antisense transcripts were observed to be co-expressed under “resting” conditions.

MicroRNAs expressed in brain, and especially those predicted to interact with synaptic mRNAs,

tend to show positively correlated (rather than inverse) expression of microRNAs and their targets, due to the fact that they interact as part of larger networks including feedback and feedforward loops (Tsang et al., 2007). Further studies are needed to learn whether the members of sense-antisense pairs regulate each other in a positive or inverse fashion, particularly under dynamic conditions or disease conditions, and particularly within specialized cytoplasmic compartments such as dendritic spines.

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Table 1. Sense-antisense transcript pairs related to Alzheimer disease pathways.

Transcript #1	Average Syn intensity #1	Average Syn enrichment #1	Transcript #2	Average Syn intensity # 2	Average Syn enrichment #2
ri|3526402A15 beta-site APP cleaving enzyme (BACE1)	24344	2.11	ri|5730407P16	6420	1.70
ri|C630003C02 reticulon 3	44319	0.95	ri|6720477H23	1446	1.30
ri|1810030E20 similar to CGI-118 PROTEIN	2827	0.96	ri|1500041A16RAB6	2075	0.65
NM_022433.1 sirtuin 3	4662	1.03	ri|6030437B01 synembryn	3848	0.73
ri|B930022J16 TAF10 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 30 kDa	622	1.04	NM_010562.1 integrin linked kinase (Ilk)	1796	1.29
ri|1300003O07 APP binding protein 2	1000	1.18	ri|4633401I16	22687	0.98

Supplementary File – 121 pairs in Excel spreadsheet.