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Cholinergic neuron gene expression differences captured by translational profiling in a mouse model of Alzheimer's disease

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ABSTRACT

Cholinergic neurotransmission is impaired in Alzheimer's disease (AD), and loss of basal forebrain cholinergic neurons is a key component of disease pathogenicity and symptomatology. To explore the molecular basis of this cholinergic dysfunction, we paired translating ribosome affinity purification (TRAP) with RNA sequencing (TRAP-Seq) to identify the actively translating mRNAs in anterior forebrain cholinergic neurons in the TgCRND8 mouse model of AD. Bioinformatic analyses revealed the downregulation of 67 of 71 known cholinergic-related transcripts, consistent with cholinergic neuron dysfunction in TgCRND8 mice, as well as transcripts related to oxidative phosphorylation, neurotrophins, and ribosomal processing. Upregulated transcripts included those related to axon guidance, glutamatergic synapses and kinase activity and included AD-risk genes *Sorl1* and *Ptk2b*. In contrast, the total transcriptome of the anterior forebrain showed upregulation in cytokine signaling, microglia, and immune system pathways, including *Trem2*, *Tyrobp*, and *Inpp5d*. Hence, TRAP-Seq clearly distinguished the differential gene expression alterations occurring in cholinergic neurons of TgCRND8 mice compared with wild-type littermates, providing novel candidate pathways to explore for therapeutic development in AD.

1. Introduction

Alzheimer's disease (AD), the most common form of dementia, is characterized by early stage dysfunction and late stage loss of cholinergic projection neurons in the nucleus basalis (NB) of the basal forebrain (reviewed in Kar et al., 2004; Liu et al., 2015; Mufson et al., 2008). Moreover, the extent of this dysfunction and loss of cholinergic neurons is correlated with the severity of disease (Bierer et al., 1995; Ginsberg et al., 2006; Whitehouse et al., 1981; Wilcock et al., 1982). Currently, acetylcholinesterase inhibitors represent the main treatment strategy for early management of AD symptoms (Schneider, 2013; Schneider et al., 2014). Although these inhibitors are able to rescue cholinergic neurotransmission at early stages, cholinergic neuronal dysfunction and loss continues to progress with disease (Corbett et al., 2012; Di Santo et al., 2013; Schneider, 2013). Therefore, a thorough understanding of the molecular mechanisms responsible for cholinergic neuron degeneration is vital to the development of effective therapeutics for patients with AD.

To gain insight into the molecular mechanisms underlying cholinergic neuron susceptibility in AD, single-cell RNA extraction and custom microarray using 576 probes and/or quantitative real-time PCR have been used to profile certain functional classes of mRNAs from basal forebrain cholinergic neurons isolated by microaspiration (Counts et al., 2007, 2009; Ginsberg et al., 2006; Mufson et al., 2002) or laser capture microdissection (LCM; Ginsberg et al., 2011; Riascos et al., 2014) from AD postmortem tissue. The collective findings from these studies revealed downregulation of neurotrophin receptors (*NTRKA*, *NTRKB*, *NTRKC*); synaptic genes (*SYP*, *SYT1*); protein phosphatases (*PP1*a, *PPI*g); cathepsin D (Mufson et al., 2002); calcium binding protein *CALB2*

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(Riascos et al., 2014); and an upregulation of a7 nicotinic acetylcholine receptor (Counts et al., 2007) and several endosomal effector genes (*RAB4*, *RAB5*, *RAB7*, *RAB27*; Ginsberg et al., 2011). It was also found that surviving cholinergic neurons had preserved mRNA levels of *GLUR2*, *SOD2*, and *GLUT2* (*SLC2A2*), relative to controls (Counts et al., 2009).

To further expand on these studies, we have used translating ribosome affinity purification (TRAP) coupled with RNA-Sequencing (TRAP-Seq) to identify the mRNAs being actively translated in anterior forebrain cholinergic neurons of the TgCRND8 mouse model of AD. In TRAP, enhanced green fluorescent protein (EGFP)—tagged ribosomal protein L10a is fused to a bacterial artificial chromosome gene promoter to give cell-type specific expression in vivo. Thus TRAP allows immunoaffinity purification of polysomal-bound mRNAs from neuron subtypes using the EGFP tag (Doyle et al., 2008; Heiman et al., 2008, 2014). We have used bacterial artificial chromosome-TRAP mice in which EGFP-L10a is expressed in cholinergic neurons through use of the choline acetyltransferase (*Chat*) promoter (*Chat*-bacTRAP mice) and crossed these with TgCRND8 mice, which express the double mutant human amyloid precursor protein harboring the Swedish (K670N, M671L) and Indiana (V717F) mutations (hAPP695) under the control of the hamster prion promoter resulting in widespread deposition of amyloid-β (Aβ) plaques in the brain (Chishti et al., 2001). To characterize cholinergic neurons in the active process of neurodegeneration, TRAP-Seq was performed on 28-week-old TgCRND8 mice when cholinergic neuron dysfunction and loss is apparent (Bellucci et al., 2006). TRAP-Seq revealed previously the unidentified pathway changes associated with cholinergic dysfunction, including the downregulation of neurotrophin signaling, oxidative phosphorylation, and RNA processing transcripts. In addition, an upregulation of transcripts related to axon guidance, glutamatergic receptors, calcium and kinase signaling was observed. Interestingly, the AD-risk genes *Sorl1* (Rogaeva et al., 2007) and *Ptk2b* (Dourlen et al., 2017; Lambert et al., 2013) were upregulated in TRAP-Seq. In contrast, sequencing the total transcriptome of the anterior forebrain demonstrated the upregulation of immune system pathways, cytokine signaling, and the transcripts for genes implicated in AD, including *Tyrobp* (Zhang et al., 2013), its putative receptor *Trem2* (Guerreiro et al., 2013; Jonsson et al., 2013), and *Inpp5d* (Lambert et al., 2013). Hence, TRAP-Seq profiling has clearly distinguished the molecular changes occurring in degenerating cholinergic neurons from the total transcriptome of the anterior forebrain of TgCRND8 mice, uncovering key pathways that can be used to generate testable hypotheses for therapeutic development in AD.

2. Methods and materials

2.1. Mouse breeding and genotyping

All protocols were conducted in accordance with the Canadian Council on Animal Care and approved by the University of Toronto Animal Care Committee. The *Chat*-bacTRAP (DW167) mouse line generously provided by Nathaniel Heintz (Rockefeller University) was re-derived on a C57BL/6 mouse background by the Jackson Laboratory (Bar Harbor, Maine) as described previously (Heiman et al., 2014). The *Chat*-bacTRAP mouse line was bred to homozygosity in-house (MacNair et al., 2016) to simplify breeding. The TgCRND8 mouse line was maintained as previously described (Chishti et al., 2001). Homozygous *Chat*-bacTRAP mice were crossed with heterozygous TgCRND8 mice to generate double transgenic TgCRND8:*Chat*-bacTRAP mice and *Chat*-bacTRAP littermates. Genotypes of all animals were confirmed by PCR (primer sets shown in Supplemental Table 1).

2.2. Western blot

To validate the TRAP procedure, the input, unbound, and EGFP immunoprecipitates from homogenates of the anterior forebrain of heterozygous *Chat*-bacTRAP mice and non-transgenic wild-type littermates were electrophoresed on 10% (w/v) sodium dodecyl sulfate-polyacrylamide gels. The separated proteins were transferred to polyvinylidene fluoride membranes, which were then blocked for 1 hour at room temperature in blocking buffer containing Tris-buffered saline (TBS; 50-mM Tris-HCl, 150-mM NaCl, pH 7.6), 5% (w/v) skim milk powder, and 0.05% (w/v) Tween 20. Subsequently, polyvinylidene fluoride membranes were incubated overnight at 4 °C with mouse monoclonal anti-EGFP (JL-8, Clontech; 1:2000) and rabbit polyclonal anti-RPL7 (NB100-2269, Novus Biologicals; 1:2000) primary antibodies diluted in the same blocking buffer. Membranes were then washed with TBS containing 0.05% (w/v) Tween 20 then incubated for 1 hour at room temperature with either the anti-mouse horseradish peroxidase—conjugated (NA931, VWR; 1:5000) or anti-rabbit horseradish peroxidase—conjugated (NA934, VWR, 1:5000) secondary antibodies diluted in blocking buffer. Finally, the membranes were washed again using TBS containing 0.05% (w/v) Tween 20 and then chemiluminescent visualization was performed using Western Lighting Plus ECL (Perkin Elmer).

2.3. Free-floating immunofluorescence

First, mice were anaesthetized with ketamine/xylazine (1 mg/g) by intraperitoneal injection. Transcardial perfusion was performed with ice-cold phosphate buffered saline (PBS; 137 mM NaCl, 2.7-mM KCl, 4.3-mM Na_2HPO_4, 1.47-mM KH_2PO_4, pH 7.4) followed by ice-cold 4% (w/v) paraformaldehyde in PBS. Brains were removed and post-fixed in paraformaldehyde for 24 hours at 4 °C, then cryoprotected by immersion in PBS with 30% (w/v) sucrose, and stored at 4 °C until sectioning. 40 μm sagittal sections were cut on a freezing microtome (HM430, Thermo Scientific), placed in antifreeze solution (30% glycerol, 30% ethoxyethanol, 40% PBS), and stored at −20 °C. Sagittal sectioning maximized the sampling of ChAT-positive regions of the mouse brain per section. Sections containing the vertical limb of the diagonal brand of Broca (VDB) and medial septal nucleus (MS) of the basal forebrain were rehydrated in PBS and then blocked in 10% donkey serum (EMD Millipore) in PBS with 0.4% (w/v) Triton X-100. Primary antibody incubation was performed with polyclonal goat anti-ChAT (AB144P; Abcam; 1:500) and mouse monoclonal anti-EGFP (JL-8, Clontech; 1:1000) diluted in the blocking serum at 4 °C. Following three 10-minute washes with PBS containing 0.1% (w/v) TX-100, sections were incubated in donkey anti-mouse 488 and donkey anti-goat 594 Alexa Fluor secondary antibodies (Invitrogen; 1:500) diluted in the same blocking serum for 2 hours at ambient temperatures. After three 10-minute washes with PBS, sections were mounted on positively charged slides and dried at room temperature. Coverslip mounting was performed with ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Life Technologies). Micrographs were captured using a Leica DMI6000B microscope and analyzed with Volocity 6.3 software (Perkin Elmer).

2.4. Tissue harvesting and TRAP procedure

Ribosomal and polysomal mRNA from *Chat*-expressing neurons was obtained from TgCRND8:*Chat*-bacTRAP mice (n = 3) and *Chat*-bacTRAP littermate mice (n = 2) as described previously (Doyle et al., 2008; Heiman et al., 2008, 2014; MacNair et al., 2016). Briefly, 28-week-old TgCRND8:*Chat*-bacTRAP mice and their littermate controls were anesthetized with CO_2, decapitated, and the