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Intersectin 1 contributes to phenotypes *in vivo*: implications for Down Syndrome

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Abstract

Intersectin 1 (ITSN1) is a chromosome 21 (HSA21) gene product encoding a multi-domain scaffold protein that functions in endocytosis, signal transduction and is implicated in Down Syndrome, Alzheimer's Disease, and potentially other neurodegenerative diseases through activation of c-Jun N-terminal kinase (JNK). We report for the first time that ITSN1 proteins are elevated in Down Syndrome individuals of varying ages. However, ITSN1 levels decreased in aged Down Syndrome cases with Alzheimer's Disease-like neuropathology. Analysis of a novel ITSN1 transgenic mouse reveals that ITSN1 overexpression results in a sex-dependent decrease in locomotor activity. This study reveals a link between overexpression of specific ITSN1 isoforms and behavioral phenotypes and has implications for human neurodegenerative diseases such as Down Syndrome and Alzheimer's Disease.

Keywords

scaffold protein; MAP kinase; endocytosis; signal transduction; neurodegeneration; SH3 domain; EH domain

INTRODUCTION

Down Syndrome is the most common form of chromosomal trisomy in humans with an incidence of 1 in 733 live births [1]. Triplication of HSA21 leads to a number of characteristic phenotypes including musculoskeletal defects, mental retardation, heart defects, and early-onset Alzheimer's disease neuropathology [2]. One of the earliest observed phenotypes of both Down Syndrome and Alzheimer's Disease patients are abnormally large early endosomal compartments, suggestive of defects in receptor trafficking [3]. Indeed, nerve growth factor transport is impaired in basal forebrain

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cholinergic neurons (BFCNs) in a Down Syndrome mouse model leading to degeneration of these neurons [4].

ITSN1 is a unique, multi-domain scaffold protein encoded by a gene located at 21q22.1-q22.2. ITSN1 mRNA is elevated in Down Syndrome brains [5]. ITSN1 consists of two major splice products, ITSN1-short (S) and long (L). Both isoforms possess two Eps15 homology domains, a coiled-coil region, and five Src homology 3 domains; however, ITSN1-L possesses an extended C-terminus encoding a Dbl homology domain, Pleckstrin homology domain, and a C2 domain. The DH-PH modules function in concert as a guanine nucleotide exchange factor that specifically activates the Cdc42 GTPase [6].

Several lines of evidence suggest a link between Down Syndrome neuropathology and ITSN1 (reviewed in [6]). First, ITSN1 acts as an endocytic scaffold regulating the assembly of clathrin-coated pits and alterations to its expression may contribute to the observed endocytic defects in Down Syndrome. Second, ITSN1 overexpression in model systems results in defective receptor regulation. Third, ITSN1 enhances activation of the JNK-MAPK pathway. As a specific initiator of the apoptotic response, JNK has been implicated in neurodegeneration [7]. Fourth, whole genome expression analyses reveal that ITSN1 is one of the most highly induced transcripts in Alzheimer's Disease brains [8–10], consistent with the development of an Alzheimer's Disease-like neuropathology in Down Syndrome patients above the age of 40 years [2]. Finally, ITSN1 overexpression enhances neurodegeneration *in vivo* in a *Drosophila* model for polyglutamine expansion diseases [11]. These effects involve the JNK pathway and support the notion that ITSN1 and JNK mediate the response of neurons to toxic stress. Together, these data suggest that ITSN1 may contribute to the sequelae of Down Syndrome.

ITSN1 expression is widely reported to be elevated in Down Syndrome; however, only a single study has examined this issue, focusing specifically on ITSN1 mRNA levels [5]. Given this paucity of data and lack of information on ITSN1 protein levels in Down Syndrome individuals, we examined the expression of ITSN1 proteins in normal and Down Syndrome cases. Our study represents the first analysis of ITSN1 protein levels throughout the human lifespan. Finally, we describe the initial characterization of a brain-specific ITSN1-S transgenic mouse which exhibits specific behavioral phenotypes. In conclusion, our findings indicate that ITSN1 proteins are overexpressed in Down Syndrome brains and may contribute to altered behavior *in vivo*.

MATERIALS AND METHODS

Subjects

Frozen tissue blocks from the midfrontal cortex were obtained from a total of 25 individuals (15M, 10F) with Down Syndrome ranging in age from 5 months to 62 years of age. A series of 16 control cases (8M, 8F) ranging in age from 6 months to 67 years were used for comparison. Down Syndrome and young or non-demented aged control cases were obtained from two sources: (1) the UCI-ADRC Brain tissue repository and; (2) NICHD Brain and Tissue Bank for Developmental Disorders.

Tissue isolation

ITSN1-S transgenic mice were generated as described in Supplemental Digital Content, Supplemental Digital Content 1, http://links.lww.com/WNR/A147. Mice were sacrificed and brains removed, placed in ice cold PBS, and brain regions isolated by microdissection. Samples were placed in PLC lysis buffer [12] supplemented with 1 mM sodium vanadate, 10 ug/ml leupeptin, 1 mM PMSF, 1 mM benzamidine, and 10 ug/ml aprotinin, homogenized, then centrifuged at 14000 RPM at 4°C to remove insoluble debris. Protein

concentrations were determined by BCA protein assay (Pierce). For human tissues, a frozen sample of frontal cortex was homogenized in PLC-lysis buffer with a polytron and then incubated at 4°C on a nutator for 20–30 min to enhance solubilization of proteins. Samples were then centrifuged and processed as above.

Antibodies and Western blot analysis

The ITSN1 antibody has been previously described [13]. Antibodies to neuron-specific tubulin (Tuj1) and the hemagglutinin epitope (HA.11) are commercially available (Covance). Western blot analyses were performed as described [12]. Differences in the levels of ITSN1-S and ITSN1-L in patient samples were determined by densitometry using ImageJ software (NIH) and normalized to the level of tubulin in each sample. The relative expression of ITSN1-S and ITSN1-L was then compared between samples of various ages.

Locomotor activity

A computerized AccuScan 12 Animal Activity Monitoring System (Columbus Instruments, Columbus, OH) assisted by VERSAMAX software (AccuScan Instruments, Columbus, OH) monitored locomotor activity in mice [14]. Each activity cage consisted of a Perspex box (20 × 20 × 20 cm) surrounded by horizontal and vertical infrared sensor beams. The stereotypic time as a measure of the time the animal spent in stereotypic activity such as grooming or head bobbing and the locomotor activity, including horizontal activity and center distance of transgenic mice was recorded between 1:00 and 3:00 p.m. in the room housing the mice.

RESULTS

ITSN1 mRNA levels are elevated in Down Syndrome [5]. However, little data exists on the expression of ITSN1 proteins in human brains. Therefore, we examined the expression of ITSN1 proteins in frontal cortex samples from normal and Down Syndrome cases (Table 1, Supplemental Digital Content 2, http://links.lww.com/WNR/A148). The two major ITSN1 isoforms, ITSN1-S and ITSN1-L, were variably expressed in both normal and Down Syndrome cases (see Figure 1, Supplemental Digital Content 3, http://links.lww.com/WNR/A149). Comparison of ITSN1-S and ITSN1-L levels between normal and Down Syndrome patients of varying ages indicated that ITSN1-S levels were consistently elevated in Down Syndrome cases (p=0.023) (Figure 1A). Although ITSN1-L levels were elevated in Down Syndrome vs control, this difference was not significant (p=0.12). Since Down Syndrome patients above the age of 40 invariably develop an early onset Alzheimer's Disease-like neuropathology [2], we asked whether ITSN1 levels varied as a function of age as well as genotype (control vs Down Syndrome) (Fig. 1B). For this analysis, Down Syndrome and control cases were grouped as being under age 40 or age 40 and older as this is the typical age at which individuals with Down Syndrome have sufficient neuropathology for a diagnosis of Alzheimer's Disease. There did not appear to be a significant variation in ITSN1 levels with age in control samples although Down Syndrome cases under the age of 40 years exhibited elevated levels of both ITSN1-S and ITSN1-L (p=0.021 and 0.035, respectively) (Fig. 1B). Interestingly, ITSN1 levels decreased in the older vs younger Down Syndrome cases. To determine whether there was a correlation between ITSN1 levels and Alzheimer's Disease pathology in the Down Syndrome cases, we stratified samples into control, Down Syndrome, and Down Syndrome with a concurrent diagnosis of Alzheimer's Disease (DS+AD) (Fig. 1C). Both ITSN1-S and ITSN1-L levels are elevated in Down Syndrome patients (p=0.01 and 0.026) (Fig. 1C). However, ITSN1-S and ITSN1-L levels are reduced in Down Syndrome+Alzheimer's Disease cases compared to Down Syndrome without Alzheimer's Disease and these differences are statistically significant (p=0.026 and 0.015, respectively). Using an independent set of brain samples isolated for cases of similar age but harvested at varying times following death, we did not

observe a systematic contribution of the time between tissue harvesting and death (post-mortem interval, PMI) to the variability in ITSN1-S (Spearman r=−0.61 p=0.14) or ITSN1-L (Spearman r=−0.90 p=0.85) levels across cases (see Figure 2, Supplemental Digital Content 3, http://links.lww.com/WNR/A149).

These results indicate that ITSN1-S and ITSN1-L are upregulated in Down Syndrome brains; however, the contribution of this overexpression to Down Syndrome is unknown. To determine whether ITSN1 overexpression contributes to the sequelae of Down Syndrome, we overexpressed ITSN1-S in the mouse brain. Several observations suggested that overexpression of ITSN1-S may have a greater affect on brain function than ITSN1-L. First, neurons normally express ITSN1-L, but not ITSN1-S [15,16]. Second, Down Syndrome patients over 40 years of age develop an Alzheimer's Disease-like neuropathology, and ITSN1-S was shown to be specifically overexpressed in neurons from post-mortem Alzheimer's Disease brain samples [8–10]. Finally, overexpression of ITSN1-S increases JNK activation and neurodegeneration [11].

To determine if brain-specific overexpression of ITSN1-S results in observable phenotypes, we generated mice that overexpress HA-tagged mouse ITSN1-S (HA-ITSN1-S) in the brain by crossing tet-HAITSN1 mice with CaMKII-tTA mice [17]. The CaMKII-tTA drives expression of a transgene from the Tet operon in neurons in forebrain structures with the highest levels in the striatum [17,18]. Double transgenic CaMKII-tTA:tet-HAITSN1 (hereafter referred to as CaMKII-ITSN1) mice specifically express HA-ITSN1-S in forebrain structures including the olfactory bulb, cerebral cortex, striatum/septum, hippocampus, and thalamus/hypothalamus with highest levels in the striatum (Figure 2A). Relatively low expression was detected in the olfactory bulb, thalamus, and midbrains. In contrast, HA-ITSN1-S was not detected in the cerebellum or in the pons/medulla. Endogenous ITSN1-S and ITSN1-L are ubiquitously expressed in the brains of single and double transgenic mice, with ITSN1-L as the predominant isoform. However, ITSN1-S expression was elevated relative to ITSN1-L levels in the CaMKII-ITSN1 samples (Fig. 2). Comparison of total ITSN1 levels (endogenous and HA-ITSN1-S) in the striatums of single (CaMKII and ITSN1) and double (CaMKII-ITSN1) transgenics reveals that the ratio of ITSN1-S to ITSN1-L is significantly elevated in double compared to single transgenic samples (Fig. 2B&C). These data indicate that HA-ITSN1-S constitutes a significant portion of the total ITSN1 protein in this brain region. Further, the level of ITSN1-S overexpression in the striatum of CaMKII-ITSN1 mice is comparable to the level of ITSN1-S overexpression in the Down Syndrome brain samples, which indicates that this transgenic line is biologically relevant for modeling the effects of ITSN1-S overexpression.

To initially characterize the phenotypic consequences of ITSN1-S overexpression, we measured the locomotor activity of single and double transgenics in an open field test. Male CaMKII-ITSN1 mice exhibited an overall reduction in activity compared to the single transgenic control mice (Figure 3). Double transgenics exhibited reduced horizontal activity (CaMKII-ITSN1 vs CaMKII, p=0.031; CaMKII-ITSN1 vs ITSN, p=0.020; Fig. 3A), exploratory behavior as measured by reduction in center distance (CaMKII-ITSN1 vs CaMKII, p=0.007; CaMKII-ITSN1 vs ITSN1, p=0.025; Fig. 3B), stereotypy time (CaMKII-ITSN1 vs CaMKII, p=0.037; CaMKII-ITSN1 vs ITSN1, p=0.005; Fig. 3C) and stereotypy counts (CaMKII-ITSN1 vs CaMKII, p=0.029; CaMKII-ITSN1 vs ITSN1, p=0.009; Fig. 3D). Female double transgenics did not exhibit a statistically significant difference in comparison to single transgenics (see Discussion).

DISCUSSION

ITSN1 is a critical regulator of endocytosis, synaptic transmission, and cell signaling [6]. Its localization to HSA21 in the Down Syndrome Critical Region and elevated expression in mouse models for Down Syndrome suggest that ITSN1 may contribute to the genesis and/or progression of this disorder (reviewed in [6]). Although ITSN1 transcript levels are elevated in the prosencephalon of Down Syndrome embryos [5], expression of ITSN1 proteins has not been described. We demonstrate that ITSN1 levels are indeed elevated in frontal cortex of Down Syndrome, with a clear distinction in expression of ITSN1 in younger (<40 yrs) vs older (≥40) Down Syndrome cases. ITSN1-S and ITSN1-L isoforms were elevated in younger Down Syndrome cases but in older Down Syndrome cases, levels of each isoform were similar to controls. This age stratification of cases corresponds with the appearance of an Alzheimer's Disease-like neuropathology in Down Syndrome [2]. This correlation suggests two possibilities: the decrease in ITSN1 levels may result from increased neurodegeneration in older Down Syndrome patients leading to loss of ITSN1-expressing cells; or ITSN1 overexpression may promote the loss of these neurons.

To address the biological consequences of ITSN1 overexpression *in vivo*, we created a novel transgenic mouse model in which ITSN1-S is specifically overexpressed in the forebrain. Although neurons only express ITSN1-L (reviewed in [6]), our mouse model is biologically relevant given the enhanced expression of ITSN1-S in Alzheimer's Disease brains [8–10] and the observed increases in ITSN1-S expression in Down Syndrome patients. ITSN1-S overexpression resulted in reduced locomotor activity in male CaMKII-ITSN1 mice. Although ITSN1-S overexpression did not result in statistically significant effects in females, female mice expressing tTA alone showed reduced center distance (not statistically significant, data not shown). Expression of tTA alone in the Tet system can have a phenotypic effect [19,20]. However, since the differences in phenotypes between male CaMKII-ITSN1 and control animals were statistically significant, we conclude that the reduced activity in these mice is due to brain-specific ITSN1-S overexpression. The highest level of expression of HA-ITSN1 was seen in the striatum, which regulates motor activity. Thus, ITSN1-S may disrupt normal functioning of neurons in the striatum by interfering with endocytic processes in these cells, altering the activity of various signaling pathways, or a combination of both.

Mouse models of Down Syndrome display abnormal behavior [21]. For example, Ts1Cje mice are trisomic for 83 genes including ITSN1, and these mice are hypoactive [21,22]. However, this mouse model overexpresses many genes and therefore may not be informative as to ITSN1's role in the abnormal behavioral phenotype. The consequences of elevated ITSN1 levels in Down Syndrome patients remain unclear. ITSN1 overexpression leads to endocytic defects, enhanced Ras activation, and increased receptor trafficking and degradation (reviewed in [6]). Interestingly, ITSN1-S overexpression increases JNK MAPK activation leading to enhanced neurodegeneration induced by polyglutamine expanded proteins [11]. These data suggest that ITSN1 overexpression in Down Syndrome may promote neurodegeneration through one or more of these pathways. This hypothesis is consistent with the increase in ITSN1-S transcripts seen in Alzheimer's Disease [8–10]. Thus, it is possible that ITSN1 cooperates with pathways to promote apoptosis of ITSN1-overexpressing cells resulting in an age-dependent decrease of ITSN1 in Down Syndrome. However, the role of ITSN1 in Down Syndrome may be more complex. ITSN1 regulates a novel PI3K-AKT survival pathway in neurons [12] and may provide a compensatory protective signal in Down Syndrome to counterbalance the negative effects of other overexpressed HSA21 genes as suggested for several HSA21 genes in Down Syndrome [23]. Additional studies using transgenic mice with targeted overexpression of ITSN1-L and overexpression of both ITSN1-S and ITSN1-L in neurons may provide insight into the

effects of ITSN1 in Down Syndrome. However, interpretation of such transgenic animals may be complicated given the lack of combinatorial overexpression of HSA21 gene products in single gene transgenics. Indeed, modeling the effects of three *Drosophila* homologs of HSA21 genes (ITSN1, Synaptojanin, and RCAN1) reveals distinct phenotypes in triple versus single or double transgenic flies [24].

Conclusion

Down Syndrome patients express elevated levels of ITSN1-S and ITSN1-L and exhibit a precipitous decrease in ITSN1 protein in aged Down Syndrome cases coincident with the development of Alzheimer's Disease neuropathology consistent. Additionally, we have shown that ITSN1-S overexpression in the mouse brain affects behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Human tissue was obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD. The role of the NICHD Brain and Tissue Bank is to distribute tissue, and therefore, cannot endorse the studies performed or the interpretation of results. Funding for human tissue samples was from UCI ADRC P50 AG16573, NICHD Contract No. N01-HD-4-3368 and NO1-HD-4-3383. M.K. was supported in part by the Craig Fellowship, University of Illinois College of Medicine. This work was supported in part by funding to G. P. from the NIH (MH 085999), to E.H. from the NIH (RO1 HD064993), and to J.P.O from the Intramural Research Program of the NIH, the Foundation Jerome Lejeune, the Department of Defense (PR080428), and the NIH (RO1 HL090651).

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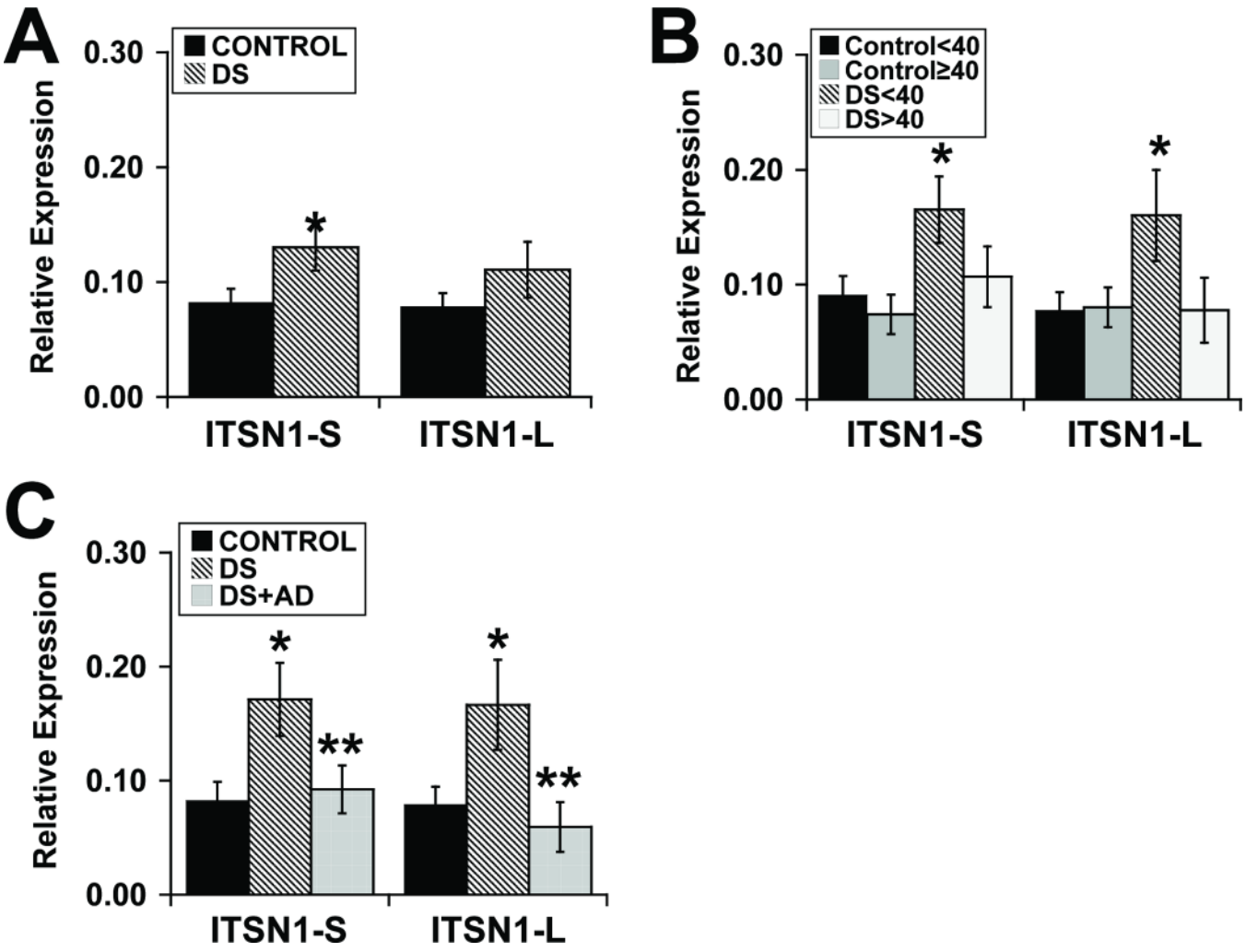

Figure 1. Expression of ITSN1 in human frontal cortex samples

(A) Relative expression of ITSN1-S and ITSN1-L was compared among all the samples. Difference in ITSN1-S levels between normal and Down Syndrome was statistically significant (p=0.023) using a Students t-test of the mean assuming unequal variance. (B) Relative expression of ITSN1 isoforms upon stratification of samples by genotype and age. Both control and Down Syndrome samples were segregated into below 40 or 40 yrs old and older. Bars represent standard error. Statistically significant differences in ITSN1-S and ITSN1-L (p=0.021 and p=0.035, respectively) in the younger Down Syndrome groupings compared to younger controls are marked, *. These differences were lost in the older Down Syndrome grouping. (C) Samples were stratified into control, Down Syndrome, Down Syndrome with diagnosed Alzheimer's Disease neuropathology (Down Syndrome +Alzheimer's Disease). Bars represent standard error. The difference in ITSN1-S and ITSN1-L levels in Down Syndrome (p=0.01 and p=0.026, respectively) vs control samples was statistically significant, *. As indicated in (C), these differences in ITSN1 levels were lost in the older Down Syndrome+Alzheimer's Disease grouping. This decrease in ITSN1 levels in Down Syndrome+Alzheimer's Disease samples vs Down Syndrome was statistically significant (**, p=0.026 for ITSN1-S; p=0.015 for ITSN1-L). Students t-test of the mean assuming unequal variance was used to calculate p values in A–C.

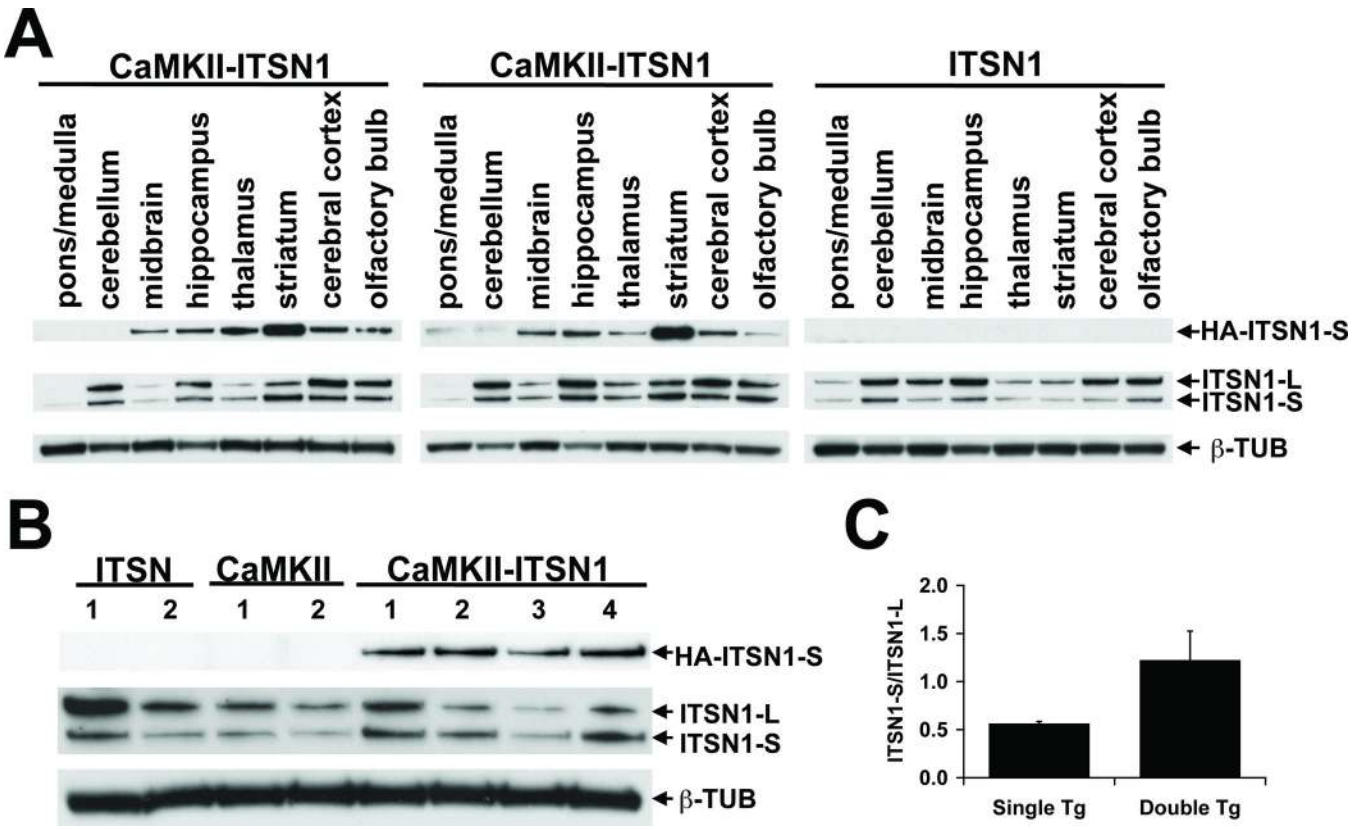

Figure 2. Expression of HA-tagged ITSN1 in brain regions of CaMKII-ITSN1 mice

(A) Top panels, HA-ITSN1 expression in the different brain regions of transgenics. Single transgenic control do not express HA-ITSN1. Middle panels, expression of endogenous ITSN1. Bottom panels, tubulin as a loading control. (B) Expression of ITSN1-L and ITSN1-S in the striatums of single transgenic controls and CaMKII-ITSN1 transgenic mice. ITSN1-L predominates in the controls, but ITSN1-S predominates in the CaMKII-ITSN1. Tubulin was used as a loading control. (C) The ratio of ITSN1-S to ITSN1-L levels in the striatums was determined from blots in (B) using NIH ImageJ. The differences in ratios were significant (p=0.022)

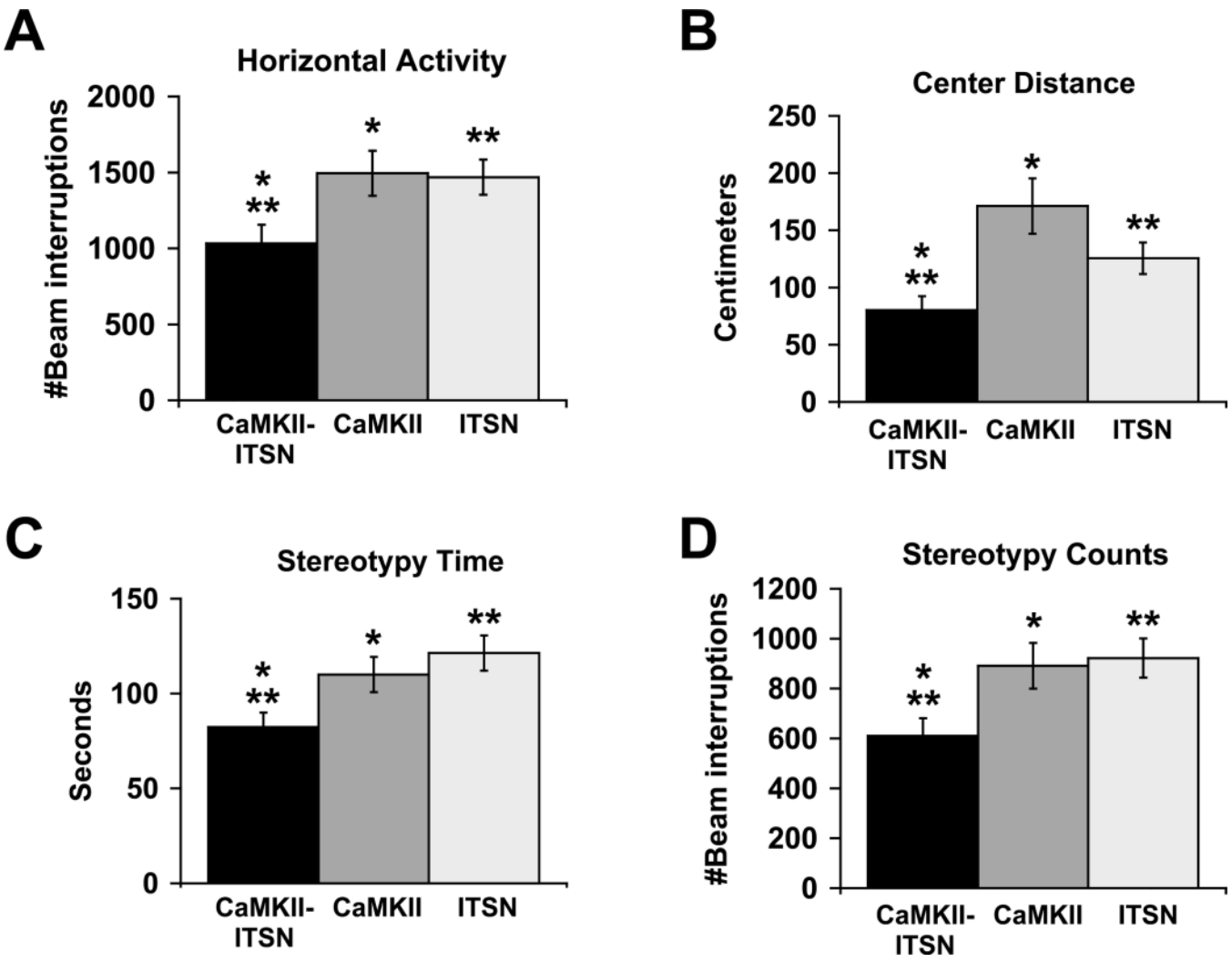

Figure 3. Reduced activity of CaMKII-ITSN1 mice

Results shown are for activity measurements during a 10 minute observation period. Male CaMKII-ITSN1 mice show reduced (A) horizontal activity (measurement of the movement of the animal in the horizontal plane of the open field chamber); (B) center distance (the total distance an animal travels in center of open field which measures exploratory behavior); (C) stereotypy time (time the animal spends in stereotypic activity such as grooming or head bobbing); and (D) stereotypy counts (the number of beam breaks that occur during stereotypic activity) compared to male single transgenic controls. Differences between CaMKII-ITSN1 and single transgenics were statistically significant for all four measures using Students t-test of the mean assuming unequal variance ($P<0.05$). Error bars show SEM. The following numbers of animals and average ages were used for this analysis: CaMKII-ITSN1: 9 M, 7.5+/−0.71 (SD) months; CaMKII: 9 M, 8.4+/−0.58 (SD) months; ITSN1: 10 M, 7.1+/−0.95 (SD) months.