

Running-induced systemic Cathepsin B secretion is associated with memory function

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Keywords

Cathepsin B, Exercise, Muscle, Memory, Hippocampus, Humans, Mice

SUMMARY

Peripheral processes that mediate beneficial effects of exercise on the brain remain sparsely explored. Here we show that a muscle secretory factor, Cathepsin B (CTSB) protein, is important for cognitive and neurogenic benefits of running. Proteomic analysis revealed elevated levels of CTSB in conditioned medium derived from skeletal muscle cell cultures treated with AMP-kinase agonist AICAR. Consistently, running increased CTSB levels in mouse gastrocnemius muscle and plasma. Furthermore, recombinant CTSB application enhanced expression of brain-derived neurotrophic factor (BDNF) and doublecortin (DCX) in adult hippocampal progenitor cells through a mechanism dependent on the multifunctional protein P11. In vivo, in CTSB knockout (KO) mice, running did not enhance adult hippocampal neurogenesis and spatial memory function. Interestingly, in Rhesus monkeys and humans treadmill exercise elevated CTSB in plasma. In humans changes in CTSB levels correlated with fitness and hippocampus-dependent memory function. Our findings suggest CTSB as a mediator of effects of exercise on cognition.

INTRODUCTION

Physical activity benefits human health, including brain function (Voss et al., 2013). In particular, exercise may maintain and improve cognition (Duzel et al., 2016). In rodents running induces changes in brain neurotransmitter, neurotrophin levels, neuronal morphology and vascularization. In addition, hippocampus-dependent memory and adult neurogenesis is enhanced (Voss et al., 2013). In humans, there is a relationship between aerobic capacity, hippocampal plasticity and memory (Duzel et al., 2016). However, peripheral mechanisms that elicit these positive effects of running remain unclear.

Peripheral factors in the blood of young animals can improve brain plasticity in aged animals (Katsimpardi et al., 2014). Given that skeletal muscle plays a pivotal role in exercise (Hawley et al., 2014), myokines (Pedersen and Febbraio, 2008) may influence neural plasticity. Indeed, overexpression of peroxisome proliferator-activated receptor-gamma co-activator (PGC-1 α) in muscle increased production of Fibronectin type III domain containing 5 (FNDC5), which is cleaved and secreted as irisin, increasing hippocampal *Bdnf* expression. Exercise elevates irisin in human plasma (Wrann, 2015). Furthermore, PGC-1 α 1 overexpression in muscle has an antidepressant-like effect by reducing entry of neurotoxic kynurenine into the brain (Agudelo et al., 2014). Moreover, spatial memory is enhanced by AICAR treatment in WT mice (Kobilo et al., 2011), but not in muscle specific AMPK mutant mice (Kobilo et al., 2014), supporting a link between skeletal muscle and cognition.

In this study, using proteomic and biochemical analyses, we found that secretory CTSB (Lemaire et al., 1997) is a myokine that is increased in plasma and gastrocnemius muscle by exercise in adult male mice. CTSB treatment of neural cells in vitro enhanced expression of DCX and BDNF. CTSB KO mice showed deficits in spatial memory, adult hippocampal

neurogenesis, dentate granule cell (GC) physiology, and hippocampal P11 levels. In Rhesus monkeys and humans treadmill exercise increased CTSB plasma levels. Moreover, in humans CTSB plasma levels were positively associated with memory. Overall, CTSB may play an important role in the beneficial effects of exercise on the brain.

RESULTS

Identification and Validation of candidates

To model effects of exercise *in vitro* we applied AMPK agonist AICAR (100 μ M) to L6 myoblast cells and analyzed the conditioned medium (CM; Figure 1A and 1B). Specifically, L6 myoblast cells were differentiated for 8 days and CM was collected after 6 hours of AICAR or vehicle (0.1% DMSO) treatment. CM was used for silver staining based proteomic analysis (Figure 1B). Interestingly, CM from AICAR treated cells revealed a differential protein expression pattern as compared to vehicle controls (Figure S1A). The differentially expressed bands were excised and eluted. We analyzed the peptide sequence of selected candidate proteins by mass spectrometry (Figure S1B). First, we selected the candidates based on the estimated protein size between 37 to 50 Kilo Dalton (kda) based on size marker (Bio-rad). Further evaluation was performed utilizing QSPEC (<http://www.nesvilab.org/qspect.php/>), a secretory database (http://spd.cbi.pku.edu.cn/spd_index.php), exercise microarray data sets (GDS2234) and AICAR-treated microarray data sets (GSE50873). The 37 kda, lysosomal cysteine protease, CTSB which has extracellular functions was selected (Figure 1A and Figure S1B).

Validation of CTSB expression after exercise

To validate CTSB as a candidate myokine that may affect the brain, we studied *Ctsb* gene expression in differentiated myoblast cells. After 8 days cells were differentiated and starved for 3 hours with serum free media and incubated with vehicle or AICAR (100 μ M) at the indicated

time points (Figure 1C). There were significant differences in *Ctsb* gene expression between time-points ($F_{(4,15)}=32.91$, $p<0.0001$). Specifically, short-term treatment (3 hours) of AICAR increased *Ctsb* mRNA levels ($p<0.0001$; Figure 1C). However, there was no increment in intracellular protein levels after treatment of AICAR as measured by western-blot (WB) analysis (Figure 1D). CTSB is known to be secreted (Lemaire et al., 1997). Therefore, extracellular protein levels were measured by ELISA. CTSB was significantly increased in differentiated L6 muscle cell lines after 100 μ M of AICAR treatment at 6 hours ($t_{(6)}=3.67$, $p<0.01$) and at 12 hours ($t_{(6)}=2.86$, $p<0.03$), (Figure 1E). Furthermore, analysis of plasma samples from mice (n= 6~8 per group) that were running for 3, 14 or 30 days showed changes in CTSB levels ($F_{(5,35)}=4.64$, $p<0.0024$). Specifically, plasma CTSB increased after 14 days and 30 days ($p<0.044$; $p<0.0008$, respectively) compared to controls (Figure 1F). In addition, *Ctsb* gene expression and protein amount was evaluated in muscle and other peripheral tissues derived from long-term (30 days) voluntary wheel running mice. *Ctsb* mRNA ($t_{(14)}=2.613$, $p<0.021$) and protein ($t_{(10)}=6.429$, $p<0.03$) levels increased in the gastrocnemius skeletal muscle (Figure 1G and 1H). *Ctsb* mRNA expression was unaltered in soleus, white adipose tissue, liver (Figure S1C), and frontal cortex (Figure S1D), and decreased in the spleen of running mice ($t_{(14)}=3.682$, $p<0.0025$; Figure S1C). These findings suggest that running results in CTSB secretion from skeletal muscle.

Behavior in sedentary and running CTSB KO mice

Increased levels of CTSB in plasma and skeletal muscle after running led us to evaluate behavior in male CTSB KO and WT littermate mice, housed under control (WT-S, KO-S) or running (WT-R, KO-R) conditions (n=7-9 per group). The running distances did not differ between groups [WT-R (2735 ± 196 m/day), KO-R (2654 ± 321 m/day), $p>0.05$].

Motor behavior

Locomotor activity in the open field was examined over 30 min. Total distance travelled did not differ between groups (Figure 2A). In addition, no difference between the groups was observed in the latency to fall in the rotarod test (Figure S1E).

Mood related behaviors

The forced swim test was used to test depression-like behavior. There was a significant main effect of genotype on immobility time ($F_{(1,27)}=8.62$, $p<0.007$; Figure 2B). Immobility time was reduced in the WT-R as compared to KO mice ($p<0.01$). Mice were also tested in the sucrose preference test to evaluate anhedonia (Figure S1F). In addition, the elevated plus maze test was performed to assay anxiety (Figure S1G). There was no difference between the groups consistent with previous research in male KO mice (Czibere et al., 2011).

Spatial memory

Mice ($n=7-9$ per group) were trained in the Morris water maze. There was no difference between the groups in acquisition of the task ($p>0.05$), (Figure 2C). After the last training day, probe trials were conducted 24h and 48h later to evaluate retention of spatial memory. The WT-R group preferred the platform quadrant compared to all other quadrants in both probe trials at 24h ($F_{(3,32)}=14.38$, $p<0.0001$) and 48h ($F_{(3,32)}=7.58$, $p<0.0006$). The WT-S group showed target preference at 24h ($F_{(3,32)}=11.54$, $p<0.0001$) but not at 48h ($p>0.05$). The KO groups did not exhibit target preference (Figure 2D).

Adult hippocampal neurogenesis

Running-induced adult neurogenesis in the dentate gyrus of the hippocampus is positively associated with memory (Voss et al., 2013). Running did not improve memory in the KO mice. There was a reduction in immature adult-born DCX⁺ Type C cells in KO mice, main effect of genotype ($F_{(1,28)}=7.735$, $p<0.0074$; Figure 2E). DCX⁺ Type D cell counts showed main effects of

running ($F_{(1,28)}=20.224$, $p<0.0001$) and genotype ($F_{(1,28)}=13.438$, $p<0.0005$; Figure 2F). Type D cells were increased in the WT-R group as compared to all other groups ($p<0.0002$). DCX staining is shown for each group (Figure 2G).

Electrophysiological recordings from dentate GCs

To determine whether CTSB KO affects memory function by changing physiological properties of mature dentate granule GCs, patch-clamp recordings were made of developmentally-born GCs (Nowakowski and Rakic, 1981). To evaluate GABAergic inhibitory transmission, recordings of miniature inhibitory postsynaptic currents (mIPSCs) were performed. mIPSCs frequency was reduced in cells ($n=15$) derived from KO mice as compared to cells ($n=12$) from WT ($t_{(25)}=3.388$, $p<0.005$), suggesting reduced inhibitory neurotransmission onto GCs (Figure 2H-L). Examination of intrinsic properties of mature GCs revealed a more depolarized resting membrane potential in KO (-82.8 ± 2.2 mV) as compared to WT (-88.4 ± 0.49 mV) cells ($t_{(17)}=2.62$, $p<0.02$). Other intrinsic properties, such as input resistance, membrane time constant and capacitance did not differ between groups ($P>0.05$).

Permeability of CTSB across the Blood-Brain-Barrier (BBB)

To test whether CTSB can cross the BBB rCTSB (50 μ g per mouse) or vehicle (distilled water) was injected intravenously (i.v.) into CTSB KO mice. Fifteen minutes after i.v. CTSB injection, there was a significant increase in blood (104.21 ± 4.11 ng/ml vs control 2.25 ± 2.20 ng/ml; $t_{(4)}=21.87$, $p<0.0001$) and tissue derived from whole brain (8.17 ± 0.90 ng/ml vs control 1.005 ± 0.46 ng/ml; $t_{(4)}=7.08$, $p<0.021$) CTSB levels.

CTSB enhances DCX and BDNF levels in aNPCs

Running increases hippocampal *Ctsb* mRNA (Figure 3A) and adult neurogenesis. Therefore, we applied exogenous CTSB to hippocampal progenitor cells with different dosages for 24 hours.

There was no effect on proliferation (live cell portion) or survival of aNPCs compared to controls (Figure S2A and S2B). We further utilized neurogenesis pathway specific PCR arrays (SAbioscience) to screen for genes regulated by rCTSB treatment in the aNPCs. Of the 86 genes, 15 genes exhibited a consistently changed expression level with 24 hour treatment of 100 ng/ml rCTSB as compared to basal differentiation media (Figure 3B and Table S1). Two genes relevant to neurogenesis, *Dcx* and *Bdnf*, were selected for validation of PCR array screening. *Dcx* mRNA ($F_{(2,9)}=5.74$, $p<0.03$) and *Bdnf* mRNA expression increased ($F_{(2,6)}=75.88$, $p<0.0001$) with rCTSB (100ng/ml) treatment, respectively (Figure 3C and 3D). Specifically, *Dcx* mRNA expression after 48 hours of rCTSB was elevated ($p<0.008$). Furthermore, 24 hours of rCTSB increased *Bdnf* mRNA compared to control ($p<0.0001$; Figure 3C and 3D). Administration of rCTSB (10 and 100 ng/ml) for 24 hours increased DCX ($F_{(4,10)}=9.03$, $p<0.0023$) and BDNF ($F_{(4,10)}=5.69$, $p<0.012$) levels in aNPCs (Figure 3E).

Involvement of P11 protein in CTSB effects on neuronal cells

Exercise increases hippocampal P11 (S100A10) expression (Sartori et al., 2011). In PC12 cells P11 level was enhanced by rCTSB ($F_{(4,10)}=6.72$, $p<0.007$; Figure 3F, Figure S2D), while hippocampal P11 was reduced in KO mice ($t_{(7)}=2.41$, $p<0.05$; Figure 3G). In addition, 12 ($p<0.002$) and 24 hours ($p<0.041$) of rCTSB (100ng/ml) increased *Dcx* mRNA compared to control (0 hour; Figure S2C). P11 knockdown using siRNA altered rCTSB treatment effect on DCX in PC12 cells ($F_{(1,8)}=9.36$, $p<0.02$). rCTSB elevated DCX levels in control but not P11 knockdown conditions (Figure 3H).

CTSB plays a role in cancer cell migration (Olson and Joyce, 2015). Five hours of rCTSB treatment affected PC12 cell migration ($F_{(5,30)}=2.55$, $p<0.048$). Specifically, rCTSB (2 ng/ml) increased PC12 cell migration ($p<0.003$; Figure 3I). There was also a significant

interaction between P11 siRNA transfection and rCTSB treatment ($F_{(3,16)}=5.01$, $p<0.012$) on PC12 cell mobility (Figure 3J). rCTSB did not enhance migration in P11 knockdown PC12 cells (Figure 3J and Figure S2E).

P11 is associated with cholesterol-rich platforms on endosomal membranes (Morel and Gruenberg, 2007) and CTSB is also involved in peripheral cholesterol absorption (Wong et al., 2013). Hippocampal 24-hydroxycholesterol levels, the form of brain specific cholesterol, was diminished in CTSB KO mice compared to WT ($t_{(10)}=2.55$, $p<0.03$; Figure 3K).

Treadmill training increases CTSB plasma levels Rhesus monkeys and humans

We evaluated CTSB levels in Rhesus monkeys and humans. In monkeys CTSB plasma levels were significantly greater in the exercise group (4 months of treadmill training) compared to control subjects ($p<0.02$; Table S2A). In humans we analyzed two forms of Cathepsin, L (CTSL) and CTSB. CTSB plasma levels in the training group differed significantly from control after four months of treadmill exercise ($p<0.048$). There was no effect on CTSL. In addition, Pearson correlation analyses across groups revealed a positive correlation between fitness increase (percentage change of VO_2 -VT) and changes in CTSB level after four months of treadmill exercise ($r=0.44$; $p<0.016$; Figure 4A, Table S2B).

Cognition and CTSB plasma levels in humans

Human subjects were tested in complex figure (CF) drawing recall test. There was a positive correlation between pre-post differences in CTSB plasma levels and late complex-object recall score (CF-score) changes ($r=0.37$; $p<0.01$, one-tailed; $p<0.05$, two-tailed). Partial correlations with VO_2 -VT as a control variable eliminated the correlation between CTSB and the CF-score ($r=0.296$; $p=0.16$) indicating that the relationship between CTSB and complex-figure recall was dependent on aerobic fitness (Figure 4B, Table S2B).

DISCUSSION

Benefits of exercise for brain function depend on central and peripheral factors. Candidate myokine CTSB may be important for brain plasticity. In vitro, AMPK activation elicited CTSB secretion in skeletal muscle cells. In vivo, exercise elevated CTSB plasma levels and hippocampal *Ctsb* gene expression, suggesting both direct and indirect CTSB effects on brain function. *Ctsb* gene KO precluded exercise induced enhancement of retention of spatial memory and adult neurogenesis, and reduced inhibitory transmission onto dentate GCs and decreased hippocampal P11, a protein needed for CTSB effects on neuronal differentiation and migration. In primates, treadmill training elevated CTSB plasma levels, and may contribute to exercise-induced memory benefits in humans.

Lysosomal cysteine protease CTSB is ubiquitously expressed throughout the body (Turk et al., 2012). High levels are found in multiple human cancers (Aggarwal and Sloane, 2014). The role of CTSB in normal physiology has remained unexplored. CTSB was increased in plasma after long-term training in mice, Rhesus monkeys and humans. Voluntary wheel running in mice elevated CTSB in plasma and gastrocnemius muscle, but not in other organs. Long-term exercise can cause muscle injury and inflammation. Transport and breakdown of amino acids and activation of the immune response are required for muscle repair. Indeed, gastrocnemius tenotomy upregulated CTSB and L activity (Harris and Baillie, 1990). Our time-course of CTSB increase is consistent with studies indicating that muscle regeneration after exercise-induced lysosomal activation takes several weeks (Salminen et al. 1984).

Our study suggests that CTSB is a myokine that can cross the BBB. However, the role of CTSB in brain function has been controversial. In a transient ischemia model, a CTSB inhibitor prevented neuronal cell death (Yoshida et al., 2002). In addition, CTSB was considered as a

protease involved in cell death after brain injury (Bannerjee et al., 2015) and onset of Alzheimer's Disease (Hook et al., 2008). However, CTSB is also reportedly neuroprotective (Bendiske and Bahr, 2003) with anti-amyloidogenic properties (Mueller-Steiner et al., 2006). Furthermore, in double-KO mice lacking both CTSB and Cathepsin L (CTSL) there is brain atrophy (Felbor et al., 2002).

CTSB may mediate the benefits of exercise for brain function through several pathways. Running increased whole hippocampus *Ctsb* gene expression. Running induces hypoxia (Radak et al., 2013) which in turn may elevate brain CTSB levels (Yakovlev and Gulyaeva, 2015). This could promote clearance of neural debris (Devi and Kiran, 2004) and adult neurogenesis, a process implicated in memory function (Abrous and Wojtowicz, 2015). Running increased neurogenesis in WT but not CTSB KO mice. In vitro analyses are consistent with in vivo observations. *Dcx* and *Bdnf* levels increased after rCTSΒ administration in aNPCs. Consistently, inhibition of both CTSB and CTSL reduced hippocampal *Bdnf* expression (Bednarski et al., 1998). BDNF regulates synaptic plasticity, cell survival and differentiation (Chao et al., 2006). DCX is important for neuronal migration, a process critical for brain development (Kawauchi, 2015).

In WT, but not KO, mice running improved spatial memory. These observations are compatible with the human exercise results. Plasma CTSB, but not CTSL (Felbor et al., 2012), levels increased after 4 months of exercise and were positively correlated with fitness levels. Humans were also tested for complex figure (CF) recall, a task that is strongly dependent on the hippocampus (Vargha-Khadem et al., 1997). The positive relationship of CTSB with complex-spatial object recall (CF) was dependent on exercise-induced changes in aerobic fitness. Aerobic

activity is also associated with an increase in hippocampal volume (Duzel et al., 2016). It will be of interest to measure whether CTSB levels are correlated with hippocampal gray matter volume.

In the periphery multifunctional adaptor protein P11, Annexin A II light chain, is a known binding partner of CTSB in caveolae of human umbilical vein endothelial cells (Cavallo-Medved et al., 2009). Annexin A2 down-regulation decreased CTSB expression in human lung adenoma cells (Wang et al., 2012). In the brain, exercise increases hippocampal P11 (Sartori et al., 2011). P11 regulates serotonin (Svenningsson et al., 2013), important for exercise-induced neurogenesis (Klempin et al., 2013), as well as glutamate and GABA (Eriksson et al., 2013). Hippocampal P11 expression and inhibitory neurotransmission onto dentate granule was reduced in CTSB KO mice. Consistently, hippocampal GABA is lower in P11 KO mice (Eriksson et al., 2013). In vitro, DCX and migration of PC12 cells (Westerink and Ewing, 2008), induced by CTSB treatment was diminished by P11 knockdown. P11 is also associated with cholesterol formation (Morel and Gruenberg, 2007) and GABAergic signaling is affected by membrane cholesterol amount (Sooksawate and Simmonds, 2001). Reduced 24-hydroxycholesterol in CTSB KO hippocampi may affect cognition.

Exercise increases CTSB in mouse and primate plasma. CTSB deficiency in mice precludes benefits of running on spatial memory. In humans there is a positive correlation between CTSB levels, fitness and hippocampus-dependent memory. These findings expand our understanding of how exercise-induced peripheral factors boost brain function.

EXPERIMENTAL PROCEDURES

Subjects

For animal experiments, one-month-old C57Bl/6 male (n=64) mice were purchased from Jackson Labs. Mice were individually housed in standard conditions with food and water *ad libitum*.

For Rhesus monkey studies, monkeys [n=13 (3 female, 10 male), 6.9~ 20.7 years old] are housed individually in standard nonhuman primate caging on a 12h light/12h dark cycle.

For human studies, healthy young adults [n=43 (24 female), 19~ 34 years old] were enrolled in the study. Subjects were randomly assigned to either the training or control group, matched by gender, age and body mass index.

Cell culture

Rat L6 skeletal myoblast cells (ATCC CRL-1458, VA) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, NY) supplemented with 10% fetal bovine serum (FBS).

The aNPCs were grown in Neurobasal medium with B27 supplement (1:50), L-glutamine (1:100), EGF (20 ng/ml), bFGF (20 ng/ml) and heparin (20 ng/ml).

PC12 cells were grown in DMEM (Gibco, NY) supplemented with 10% FBS.

Molecular analyses

For qPCR analysis, total RNA was extracted from L6, aNPC, PC12 cells and various tissues using a total RNA extraction kit (Ribozol, Amresco) according to the manufacturer's manual.

For western blotting, equal amounts of protein lysates from cells and tissues were subjected to SDS-PAGE and transferred to a NC membrane (Millipore, MA). Specific signals were visualized by Odyssey (Amersham Bio-sciences).

For the ELISA assays, species-specific ELISA kits were used according to the manufacturer's specifications. The plate was read using SpectraMax Plus 384 Microplate Reader (Molecular Devices, Sunnyvale, CA).

Behavioral testing

Mice were housed individually in standard or running wheel cages. After four weeks mice were tested in the open field, rotarod, Morris water maze, forced swim test, sucrose preference test and elevated plus maze.

For detailed methods, see Supplemental Experimental Procedures.

Accession numbers

The accession number for the LC-MS/MS mass spectrometry analysis data re-reported in this paper is PeptideAtlas: PASS00879.

AUTHOR CONTRIBUTIONS

H.Y.M., E.D., H.v.P. designed experiments. M.H.Y, A.B., D.B., B.B., N.S., G.B., E.J., S.L., N.G., J.M. performed experiments. M.H.Y., A.B., D.B., B.B., N.S., E.J., E.D., H.v.P. analyzed the data. H.Y.M. and H.v.P. wrote the manuscript.

ACKNOWLEDGMENTS

This work was supported in part by the BMBF (Energl Project), National Institute on Aging, Intramural Research Program, the Korean Visiting Scientist Training Award (KVSTA to H.Y.M.) and the Deutsche Forschungs Gemeinschaft (SFB 779 TP A7). We thank Drs. Young Ah Goo and Hyung Won Choi for Proteomic analysis, Yang An for statistical advice and Linda R. Kitabayashi for image preparation.

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Figure Legends

Figure 1. CTSB as a candidate myokine. (A) CTSB is present in the conditioned media (CM) of AICAR (AIC, 100 μ M) and Vehicle (Veh, 0.1% DMSO) treated differentiated L6 myoblast cultures as indicated by WB analysis. PONCEAU staining was used as a loading control. (B) Flowchart indicating how CTSB was identified. (C, D, E) *Ctsb* gene expression and CTSB protein levels in cells and CM from cultures treated with Vehicle (0.1 % DMSO) or AICAR (100 μ M). (C) Time-course analysis showed *Ctsb* mRNA increased 3 hours after AICAR treatment. (D) WB analysis of intracellular CTSB levels at indicated time-points (hours) after treatment with AICAR (100 μ M). The graph shows the relative CTSB intensity normalized by β -actin. There was no difference in intracellular CTSB levels. E) CTSB protein levels in the CM of AICAR (100 μ M) treated cultures is increased at 6 and 12 hours as compared to control. (F-H) *In vivo* analyses of *Ctsb* gene expression and CTSB protein levels in plasma and gastrocnemius muscle tissue of sedentary (Sed) and running (Run) mice. (F) Running increased CTSB plasma levels at 14 and 30 days. (G) *Ctsb* gene expression was elevated in the mouse gastrocnemius muscle after 30 days of running. (H) CTSB protein levels are elevated in gastrocnemius muscle after 30 days of running. Specifically, WB analysis of CTSB levels and a graph showing the relative intensity normalized by β -actin. Data represent means \pm S.E.M. * $p < 0.05$

Figure 2. Behavioral analyses of CTSB KO mice and WT littermates housed under sedentary or running conditions. (A) Total distance travelled in the open-field test did not differ between the groups (WT sedentary, WT-S; WT runner, WT-R; KO sedentary, KO-S; KO runner, KO-R). (B) Forced swim test showed increased immobility time in KO compared to WT mice. (C) Water maze acquisition over 7 days did not differ between the groups. (D) Probe trials (60-sec) were performed 24 hours and 48 hours after the last training session. WT-R mice preferred the target quadrant as compared to all other quadrants in both probe trials. WT-S mice searched preferentially in the target quadrant at 24 hours but not at 48 hours. Neither KO-S nor KO-R mice showed retention of spatial memory. (E) DCX⁺ Type C cell number/section was higher in WT than KO groups. (F) DCX⁺ Type D cell number/section in the WT-R group was greater than in all the other groups. (G) Representative DG images of DCX staining. (H-L) Patch-clamp recordings from mature DG cells in acute slices derived from WT (n=3 mice, 12 cells) and KO (n=3 mice, 15 cells). (H) Averaged data from WT (n=12) and KO (n=15) cells show a significant decrease in mIPSC frequency. (I-K) There was no change in (I) amplitude (J) rise time or (K) decay tau. (L) Representative traces of whole cell patch-clamp recordings of mIPSCs from WT (green trace) and KO (blue trace) mice. Data represent mean \pm SEM. *p<0.05

Figure 3. Hippocampal *Ctsb* gene expression and analysis of CTSB function (A) Thirty days of running (R) increased hippocampal *Ctsb* mRNA levels compared to sedentary (S) controls. (B) aNPC cultures were treated with rCTSB (100 ng/mL) for 24 h and analyzed by Q-PCR arrays. (C-D) *Dcx* and *Bdnf* gene expression were among the top ten most elevated genes. RT-PCR analysis shows a significant increase in *Dcx* mRNA level after 48 hours and *Bdnf* mRNA level after 24 hours of rCTSB (100 ng/ml) treatment as compared to control (0 hour). (E) WB analysis of DCX, TrkB and BDNF protein levels in aNPCs treated with rCTSB or Vehicle (Veh, Distilled water) for 24 h. The graphs show the relative intensity normalized by β -actin. rCTSB treatment (10 and 100 ng/ml) significantly increased DCX and BDNF levels, as compared to Mock or Vehicle treatment. (F) *P11* mRNA levels in PC12 cells are elevated 6, 12 and 24 hours after treatment with rCTSB, 100 ng/ml. *Hsp90* gene was used for normalization. (G) Hippocampal CTSB and P11 levels in CTSB KO and WT littermates measured by WB analysis, β -actin was used as a control. P11 was significantly reduced in the CTSB KO. (H) Knockdown of P11 with si-RNA in PC12 cells decreased DCX protein level elevation by rCTSB (100 ng/ml), (Scr, scramble siRNA transfected cells) measured by WB analysis, β -actin was used as a control. (I, J) Effect of rCTSB (0.2, 2, 20 or 200 ng/ml) on cell migration. (I) Percentage of migrated PC12 cells treated with 5% FBS or 2ng/ml of rCTSB showed increased migration. (J) Knockdown of P11 in PC12 cells suppressed cell migration induced by rCTSB (2ng/ml) treatment for 5 hours as compared to Scr control. (K) Hippocampal 24-hydroxy cholesterol level was reduced in the CTSB KO mice. Data represent mean \pm SEM. * $p < 0.05$. Veh, vehicle (Distilled water).

Figure 4. Effects of four months of treadmill exercise on correlations between plasma CTSB, fitness and complex figure recall (CF) in humans. (A) There is a positive correlation in humans between aerobic fitness change as measured by VO₂ ventilatory threshold (VT) and CTSB plasma protein level change after 4 months of treadmill training intervention ($p < 0.016$). (B) There is a positive correlation between change in CF score and CTSB level change ($p < 0.01$, one-tailed; $p < 0.05$, two-tailed) after 4 months of treadmill training.

Figure 1

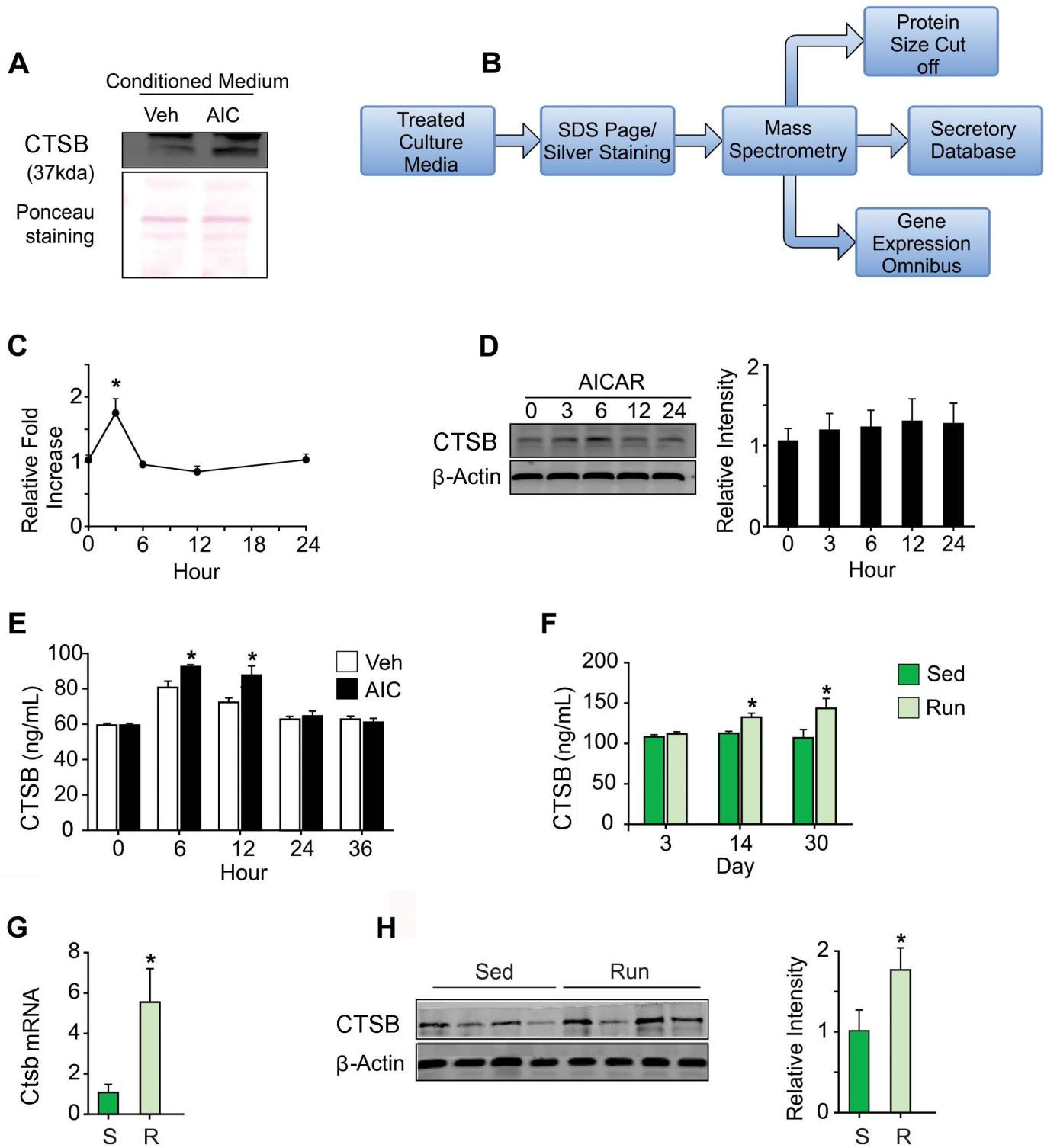


Figure 2

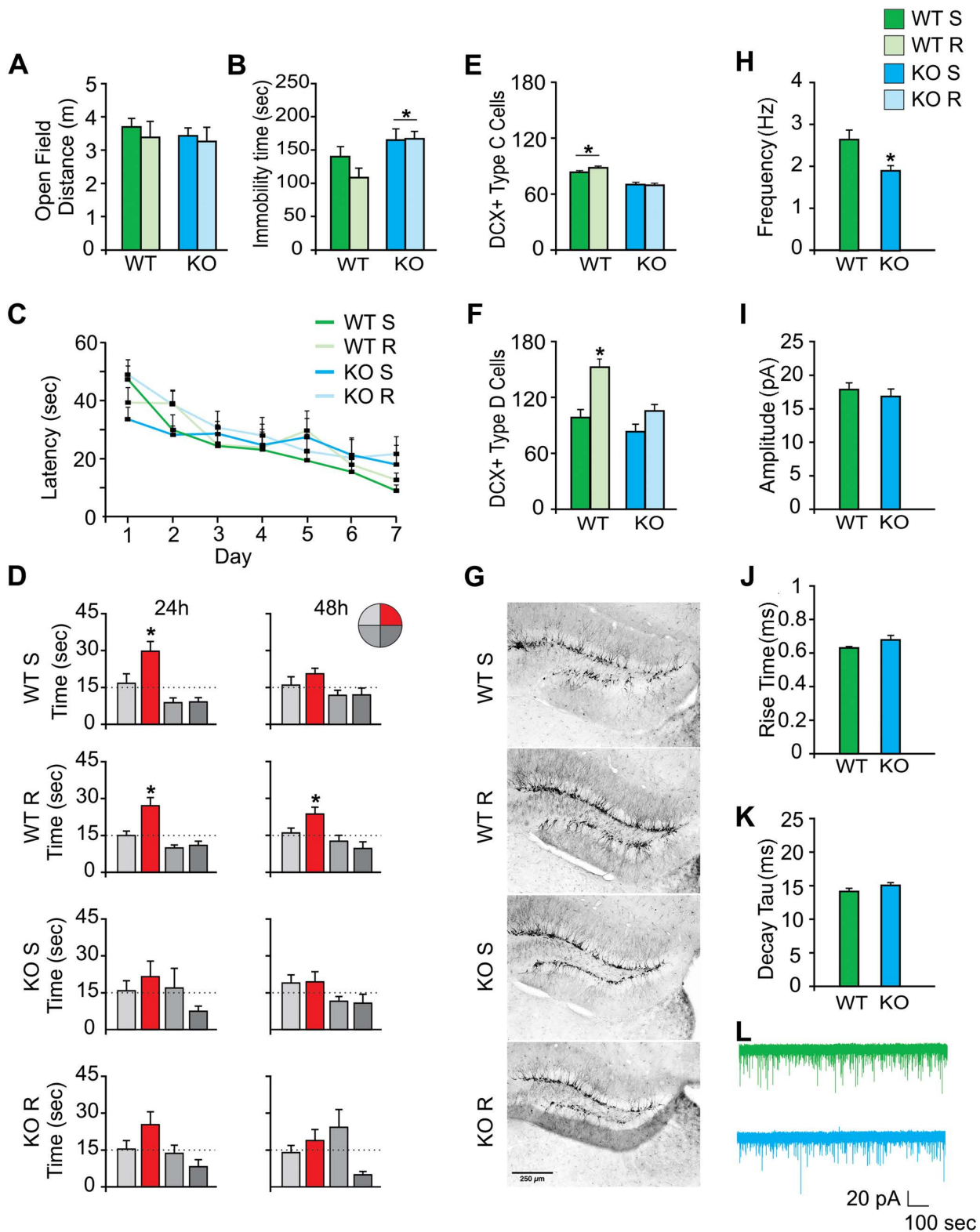


Figure 3

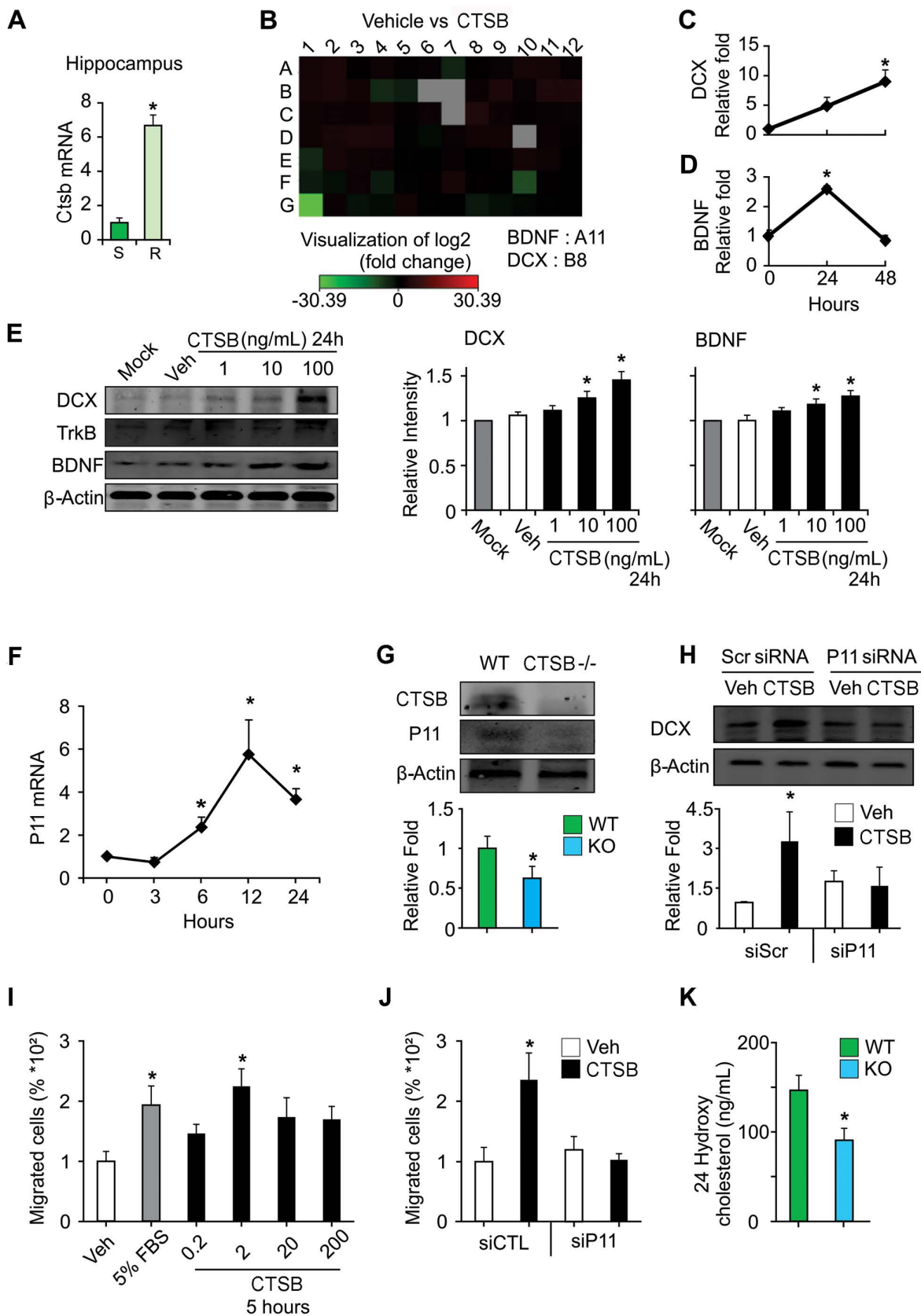
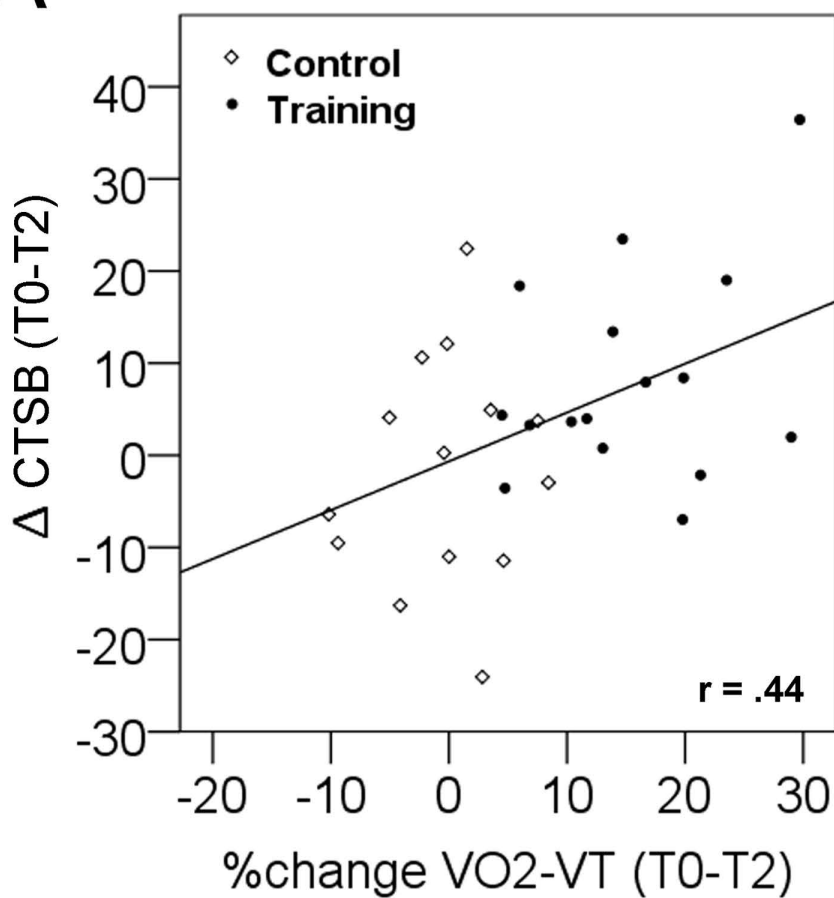
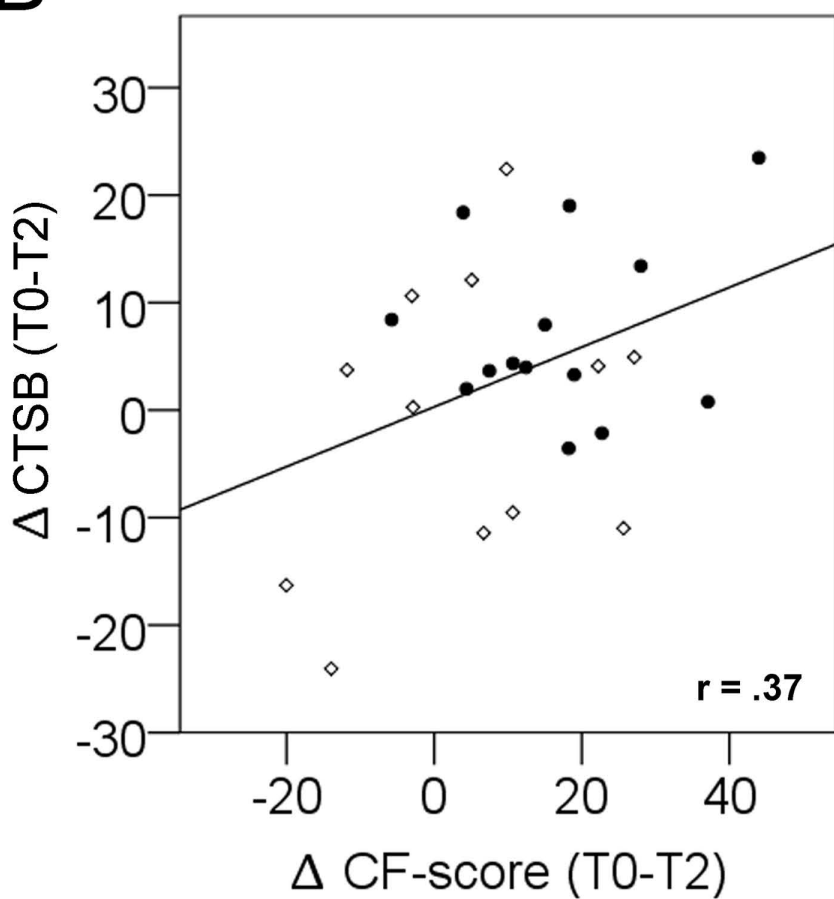


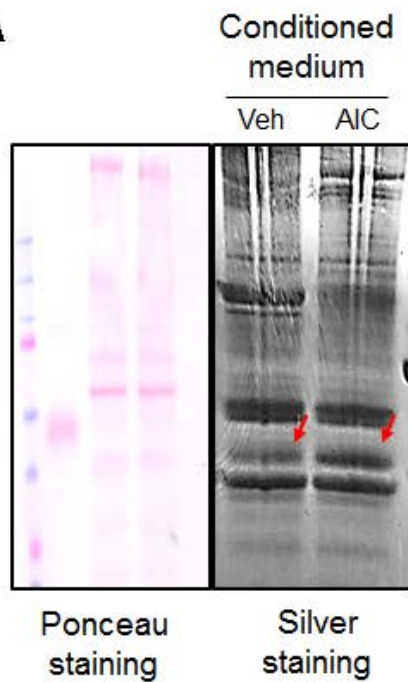
Figure 4

A



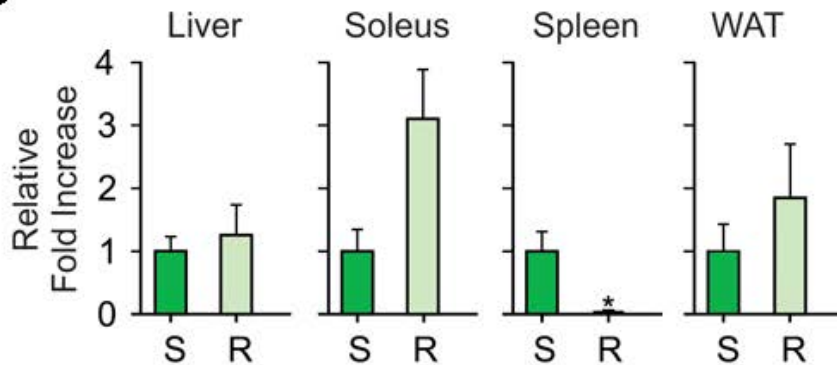
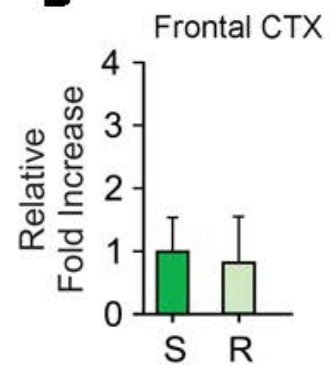
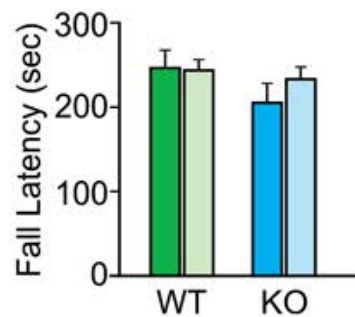
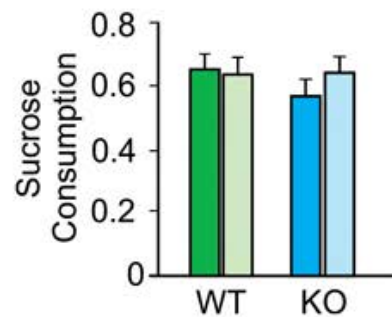
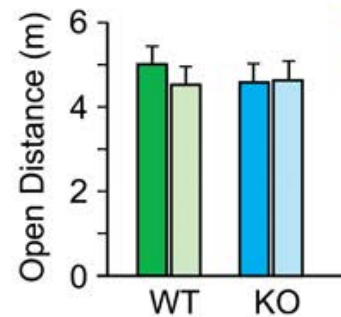
B



A**B**

AICAR modulated Secretory proteins in differentiated L6 skeletal muscle cells

Name	Protein ID	Size (Kda)	Log Fold	SPD	GDS 2234	GSE50873 (days)
Protein disulfide-isomerase A6	sp Q63081 PDIA6_RAT	44.0	5.302	N.D.	Down	N.C.
Guanine deaminase	tr Q9JK87 Q9JK87_RAT	45.4	3.197	N.D.	N.C.	N.C.
Alpha-enolase; B	sp P15429 ENOB_RAT	43.4	3.149	Sec.	N.D.	N.C.
Serpin H1	sp P29457 SERPH_RAT	41.7	3.107	Sec.	Down	N.C.
Rab GDP dissociation inhibitor beta	sp P50399 GDIB_RAT	44.5	2.553	N.D.	N.C.	N.C.
Rab GDP dissociation inhibitor alpha	sp P50398 GDIA_RAT	44.7	2.157	N.D.	N.C.	N.C.
Cathepsin B1	sp P00787 CATB_RAT, tr Q6IN22 Q6IN22_RAT	33.9	1.974	Sec.	Up	Up (3, 7, 14)
Alpha-enolase; A	sp P04764 ENOA_RAT, tr MOR5J4 MOR5J4_RAT	43.4	1.969	Sec.	N.D.	N.C.
Cathepsin D	tr Q6P6T6 Q6P6T6_RAT	40.7	1.951	Sec.	Down	N.C.
Lactadherin	sp P70490 MFGM_RAT, tr Q1PB11 Q1PB11_RAT	42.7	1.945	Sec.	N.C.	N.C.
Fascin	sp P85845 FSCN1_RAT	49.3	1.945	N.D.	Up	N.C.
Vimentin	sp P31000 VIME_RAT	46.6	1.879	N.D.	N.C.	Down (7, 14)
6-phosphogluconate dehydrogenase	sp P85968 6PGD_RAT, tr F1M9K9 F1M9K9_RAT	48.3	1.769	N.D.	N.C.	N.C.

C**D****E****F****G**

■ WT S
■ WT R
■ KO S
■ KO R

Fig. S1, related to Figures 1 and 2. Identification of secreted proteins following AICAR administration in differentiated L6 muscle cells. (A,B) Protein samples of conditioned medium (CM), derived from vehicle (Veh, 0.1% DMSO) or AICAR (AIC) treated differentiated L6 cells, were loaded on SDS-PAGE gels (lanes of Veh and AIC, respectively). The proteins were transferred to a nitrocellulose membrane and stained with Ponceau S (A, left panel). The proteins from each CM were also visualized with silver staining. Red arrow indicates the differentially expressed bands in both lanes (A, right panel). (B) List of representative proteins from LC-MS/MS analysis and additional candidate selection methods including [Label-free spectral count data analysis (<http://www.nesvilab.org/qspect.php/>)], Secretary protein database (SPD, spd.cbi.pku.edu.cn), exercise and AICAR treatment microarray Gene Expression Omnibus (GEO) datasets [Databases <http://www.ncbi.nlm.nih.gov/gds/>]: [GDS2234, GSE50873]. Not detected (N.D.); Secretary protein (Sec); Not changed (N.C.). (C,D) Effect of exercise on *Ctsb* gene expression in various tissues. (C) RT-PCR analysis of *Ctsb* mRNA in liver, soleus, spleen and white adipose tissues after 30 days of exercise. A significant decrease in *Ctsb* mRNA in the spleen ($p < 0.002$) after exercise was observed, whereas there were no changes in *Ctsb* mRNA in liver ($p = 0.67$), soleus ($p = 0.14$), white adipose tissue (WAT), ($p = 0.29$) and (D) frontal cortex (CTX), ($p = 0.81$). *Hsp90* mRNA levels were used as a control. (E-G) Behavioral analyses of male CTSB KO mice and WT littermates housed in voluntary running or sedentary conditions. (E) Rotarod latency-to-fall was not significantly changed among groups ($p > 0.05$). (F) Ratio of sucrose consumption to total amount of fluid intake in each group is shown. There were no significant changes in sucrose consumption among groups ($p > 0.05$). (G) Elevated plus maze was used to measure the anxiety of the mice. Time spent in open arms was recorded. There were no significant differences between the groups ($p > 0.05$). Data represent means \pm S.E.M. * $p < 0.05$.

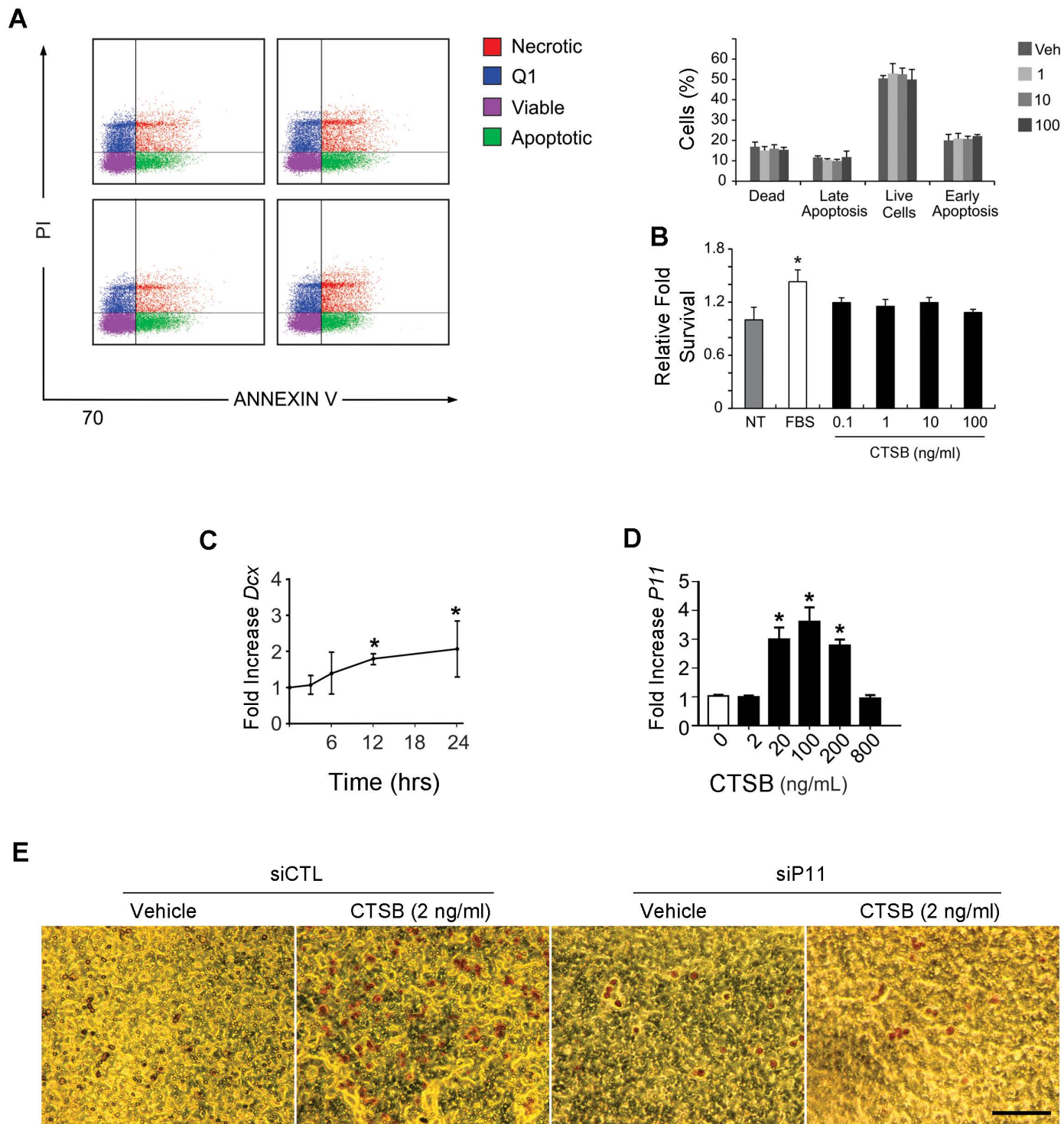


Fig. S2, related to Figure 3. Effects of recombinant CTSB on cell survival and proliferation of adult neural progenitor cells (aNPCs) and cellular function in PC12 cells. (A) FACS analysis was performed to analyze the effect of 24hour treatment of various dosages of rCTSB (1, 10, 100 ng/ml) on aNPCs proliferation and survival. (B) MTT assay was performed to analyze the effect of 24hour treatment of various dosages of rCTSB (0.1, 1, 10, 100 ng/ml) on aNPCs survival. MTT analysis indicated that FBS ($F_{(5,12)} = 4.9$, $p < 0.011$), but not CTSB enhanced survival of aNPCs compared to control. (C) Real-time RT-PCR was performed to measure mRNA expression of the neurogenesis marker *Dcx* in PC12 cells at indicated time points. CTSB significantly increased *Dcx* mRNA level at 12h compared to control (0h). (D) Real-time RT-PCR was performed to measure mRNA expression of *P11* in PC12 cells treated for 24 hours with recombinant CTSB at indicated dosages. (E) Representative images of the effect of P11 siRNA on PC12 cell migration induced by recombinant CTSB (2 ng/ml). Scale bar 50 μ m. Data represent means \pm S.E.M. * $p < 0.05$. NT, Not treated; FBS, Fetal bovine serum.

Table S1. Related to Figure 3. Neurogenesis specific qPCR array

on	Gene Symbol	<u>Fold Change (compared to control group)</u>		on	Gene Symbol	<u>Fold Change (compared to control group)</u>	
		Exp. 1	Exp. 2			Exp. 1	Exp. 2
		Fold Change	Fold Change			Fold Change	Fold Change
A01	<u>Ache</u>	1.06	3.27	D07	<u>Map2</u>	4.69	1.59
A02	<u>Adora1</u>	1.49	5.94	D08	<u>Ndn</u>	2.38	2.75
A03	<u>Adora2a</u>	1.05	0.99	D09	<u>Ndp</u>	1.16	3.23
A04	<u>Alk</u>	1.79	2.43	D10	<u>Neurod1</u>	1.03	0.62
A05	<u>Apbb1</u>	0.89	1.04	D11	<u>Neurog1</u>	2.2	1.79
A06	<u>ApoE</u>	1.53	1.4	D12	<u>Neurog2</u>	1.01	1.14
A07	<u>App</u>	0.11	0.04	E01	<u>Nf1</u>	0.23	0.02
A08	<u>ArtN</u>	1.03	0.82	E02	<u>Nog</u>	0.49	4
A09	<u>Ascl1</u>	1.16	1.55	E03	<u>Notch1</u>	2.68	2.43
A10	<u>Bcl2</u>	2.85	4.11	E04	<u>Notch2</u>	1.03	1.97
A11	<u>Bdnf</u>	2.68	3.51	E05	<u>Nr2e3</u>	1.03	0.45
A12	<u>Bmp2</u>	1.03	1.24	E06	<u>Nrcam</u>	1.03	0.53
B01	<u>Bmp4</u>	1.15	4.32	E07	<u>Nrg1</u>	1.22	1.33
B02	<u>Bmp8b</u>	1.49	5.58	E08	<u>Nrp1</u>	2.06	0.59
B03	<u>Cdk5r1</u>	0.82	2.75	E09	<u>Nrp2</u>	0.73	1.11
B04	<u>Cdk5rap2</u>	0.46	0.02	E10	<u>Ntf3</u>	1.01	3.27
B05	<u>Chrm2</u>	1.03	0.06	E11	<u>Ntn1</u>	0.32	2.43
B06	<u>Creb1</u>	1.01	0.02	E12	<u>Tenm1</u>	1.56	0.62
B07	<u>Cxcl1</u>	1.03	0.02	F01	<u>Olig2</u>	1.85	0.09
B08	<u>Dcx</u>	3.03	1.72	F02	<u>Pafah1b1</u>	3.03	0.2
B09	<u>Dlg4</u>	0.72	6.63	F03	<u>Pard3</u>	0.62	2.13
B10	<u>Dll1</u>	0.9	1.66	F04	<u>Pax3</u>	1.03	0.1
B11	<u>Drd2</u>	0.63	8.17	F05	<u>Pax5</u>	1.83	0.42
B12	<u>Dvl3</u>	1.83	7.52	F06	<u>Pax6</u>	0.83	0.98
C01	<u>Efnb1</u>	2.77	2.5	F07	<u>Pou3f3</u>	1.8	4
C02	<u>Egf</u>	2.41	2.06	F08	<u>Pou4f1</u>	1.84	0.91
C03	<u>Ep300</u>	1.03	1.87	F09	<u>Ptn</u>	1.47	0.71
C04	<u>ErbB2</u>	1.79	2.87	F10	<u>Rac1</u>	1.65	0
C05	<u>Fgf2</u>	2.91	1.07	F11	<u>Robo1</u>	1.46	0.78
C06	<u>Flna</u>	1.09	2.06	F12	<u>Rtn4</u>	1.19	0.59
C07	<u>Gdnf</u>	1.03	0.02	G01	<u>S100a6</u>	0.13	0
C08	<u>Gpi1</u>	0.81	8.82	G02	<u>S100b</u>	0.93	1.77
C09	<u>Grin1</u>	2.91	2.23	G03	<u>Shh</u>	2.83	0.19
C10	<u>Hdac4</u>	3.05	1.15	G04	<u>Slit2</u>	0	0.25
C11	<u>Hes1</u>	1.62	1.39	G05	<u>Sod1</u>	0.95	2.89

C12	<u>Hey1</u>	0.63	1.59	G06	<u>Sox2</u>	2.31	0.52
D01	<u>Hey2</u>	1.44	2.35	G07	<u>Sox3</u>	1.03	0.63
D02	<u>Hey1</u>	0.86	5.03	G08	<u>Stat3</u>	1.03	0.11
D03	<u>Il3</u>	0.87	6.92	G09	<u>Tgfb1</u>	1.57	0.22
D04	<u>Mdk</u>	0.79	5.31	G10	<u>Th</u>	2.62	0.31
D05	<u>Mef2c</u>	3.13	0.39	G11	<u>Tnr</u>	1.27	0.49
D06	<u>Kmt2a</u>	0.56	0.26	G12	<u>Vegfa</u>	3.14	0.66

Table S1, Related to Figure 3. Neurogenesis specific qPCR array. Neurogenesis pathway specific PCR array (SAbioscience) was used to screen for genes regulated by recombinant CTSB treatment (100 ng/ml) in the aNPC cultures. Of the 86 genes, 15 genes in aNPCs exhibited a consistently changed level of expression (cut-off value was 0.5 under/above fold change in two independent experiments, Exp. 1 and Exp. 2) with 24 hour treatment of 100 ng/ml recombinant CTSB as compared to basal differentiation media (Neurobasal media with 0.5% FBS) control.

Table S2, related to Figure 4. Treadmill exercise effects in Rhesus monkey and humans

(A) Monkey

	Control	Training (4 months)
CTSB (ng/ml)	169.3 ± 6.263	212.8 ± 9.869*

(B) Human

	Baseline (T0)	1 month (T1)	4 months (T2)
CTSB (ng/ml) Control	40.980 ± 1.548	36.973 ± 1.468	39.156 ± 1.648
CTSB (ng/ml) Training	36.507 ± 1.683	39.899 ± 1.995	44.393 ± 1.822*
CTSL (pg/ml) Control	96.727 ± 2.146	85.172 ± 2.824	98.891 ± 8.123
CTSL (pg/ml) Training	100.086 ± 8.614	94.813 ± 5.166	101.779 ± 5.262
VO₂_VT (ml) Control	1506.38 ± 308.47	1487.08 ± 324.25	1511.85 ± 305.22
VO₂_VT (ml) Training	1563.87 ± 356.23	1669.20 ± 344.61*	1788.27 ± 358.30*
Lactate (mmol/l) Control	4.948 ± 1.395	5.648 ± 1.708	5.398 ± 1.461
Lactate (mmol/l) Training	4.610 ± 1.382	4.208 ± 1.226	3.139 ± 1.243*

Table S2, related to Figure 4. Treadmill exercise effects in Rhesus monkey and humans.

Treadmill exercise elevates Cathepsin B (CTSB) in monkey and human plasma. (A) CTSB plasma levels in control (n=4; 1 female, 3 male Rhesus monkeys) and treadmill training (n=9; 2

female, 7 male Rhesus monkeys) groups were measured. Treadmill training increased CTSB in monkeys ($p < 0.02$). (B) CTSB and Cathepsin L (CTSL) plasma levels and fitness (VO_2 & lactate) were measured in the humans at (Baseline, T0), after 1 month (T1) and 4 months (T2) assigned to control or treadmill training groups. One-way ANOVA with repeated measures over time showed that after 4 months (T2) of treadmill exercise CTSB plasma levels increased compared to the control group ($p < 0.048$). There were also significant changes in VO_2 -VT and maximum lactate levels. The training group showed increased VO_2 from T0-T1 ($p < 0.004$) and T0-T2 ($p < 0.001$), and a decrease in maximum lactate from T0-T2 ($p < 0.001$). Comparisons for the control group revealed no significant differences between time points. Data represent mean \pm S.E.M.* $p < 0.05$.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Subjects

Mice

One-month-old C57Bl/6 male (n=64) mice were purchased from Jackson Labs. Mice were individually housed in standard conditions with food and water *ad libitum*. A week later, mice were divided into two groups (sedentary, runner) for experiments of 3, 14 or 30 days duration. Voluntary wheel running distance was recorded using Clocklab software (Actimetrics, Wilmette, IL). CTSB knockout mice, C57BL/6J × 129/SvJ genetic background, were kindly provided by Gregory J. Gores, Mayo Medical School (Halangk et al., 2000). Heterozygous offspring was obtained by breeding CTSB knockout (-/-) mice with C57Bl/6J mice. These heterozygous mice were intercrossed to generate male CTSB -/- mice (n=28), and wild-type CTSB +/+ littermates (n=27), which were used as controls. For genotyping, genomic DNA was extracted from 3mm of mouse tail with 200µl of lysis buffer (Viagen) and 5µl of proteinase K (10mg ml⁻¹). Each tail sample was incubated at 55°C for 4 hours. Thereafter, each sample was incubated at 85°C for 45 min to inactivate proteinase K activity. PCR analysis was performed with specific primers for the genotyping. For PCR annealing temperature was 57.5°C for 45 seconds.

Genotyping primers

MCB-11	F	GGT-TGC-GTT-CGG-TGA-GG	Musculus
MCB-GT	R	AAC-AAG-AGC-CGC-AGG-AGC	

Animals were maintained in accordance with the National Institutes of Health guidelines. All protocols for procedures were approved by the NIA's Institutional Animal Care and Use Committee.

Rhesus Monkeys

Housing

Monkeys [n=13 (3 female, 10 male), 6.9~ 20.7 years old] are housed individually in standard nonhuman primate caging on a 12h light/12h dark cycle, room temperature 78+/-2 degrees humidity at 60+/-20%. Monkeys always have extensive visual, auditory, and olfactory but limited tactile contact with monkeys housed in the same room. Environmental enrichment includes a rotation of floor and hanging toys and an assortment of food treats and occasional TV viewing. Monkeys are fed twice a day at approximately 7:30 am and 2:15 pm. The monkey receives a designated ration based on body size and preference of a standard monkey chow LabDiet 5038 (LabDiet, St. Louis, MO). Water is always available ad libitum.

Treadmill training

Monkeys were not “handled” while awake due to safety issues. For treadmill training, they were jumped into a transfer box and then transferred into the treadmill box. Monkeys walked 0.25 miles per day, generally 5 days a week, for 4 months.

Blood collection

Monkeys were fasted the morning of the procedure. They were anesthetized with 10 mg/kg intramuscular (IM) Ketamine. Blood was collected into vacutainer tubes, spun, serum and plasma was aliquoted and frozen at -80° C. Blood collection occurred between 8 and 10 am.

Human subjects

Physically inactive healthy young adults [n=43 (24 female), 19~ 34 years old] were enrolled in the study. Subjects were randomly assigned to either the training or control group, matched by gender, age and body mass index. Their fitness levels were corroborated by an initial fitness test to assess oxygen consumption at respiratory compensation [(VO₂-RC (ml/kg)]. A total of 56

participants were screened and 13 were excluded because they reported to engage in sports to improve or sustain cardiovascular fitness (n=12), or did not fulfill the criteria of low physical fitness, indicated by $VO_2\text{max}$ (n=1). All subjects signed written informed consent and received monetary compensation for participation. The study protocol was approved by the ethics committee of the University of Magdeburg, Germany.

Treadmill training

Subjects in the training group received individually optimized treadmill training for 16 weeks and a sports scientist supervised the intervention. The training group (n=20) received 3 sessions per week of moderate to intense aerobic exercise interval training at 70-90% of maximum heart rate for 45-75 min, while the control group (n=23) came in twice a week and walked for 10-15 min at 50% of maximum heart rate. All subjects in the training group received the same training plan adapted to their individual heart rate zones. Before intervention and after 4 weeks of intervention, the individual heart rate (HR) zones for training were estimated in relation to heart rate values at the individual anaerobic threshold during fitness test measures.

Fitness assessment

Consumption of oxygen at ventilatory threshold ($VO_2\text{-VT}$) was assessed by graded maximal exercise testing on a treadmill ergometer (HP-COSMOS, QUARK CPET). From an initial speed of 3km/h the intensity increased every 2 min up to a maximum of 6.5km/h, and also with a rise in the steepness of treadmills from 0%-18%. The end of the exercise training was reached when an exchange ratio exceeded the value of 1.1. As a precaution against possible health complications the subjects were not requested to proceed until volitional exhaustion or $VO_2\text{-peak}$ values. All subjects reached the respiratory compensation point, which was sufficient for estimation of individual target heart rate zones for aerobic exercise training and for measurement of the

volume of O₂ at ventilatory threshold (VT). VO₂-VT was used as a measure of fitness changes (Gaskill et al., 2001). In addition, starting at resting state, blood samples from the ear lobe were taken every 2 min during the fitness test and also after 2 min following maximum intensity in order to assess lactate concentration to verify individual anaerobic threshold levels for training regimen (Biosen C Line, EKF DIAGNOSTIC, Magdeburg).

Behavioral task design

The subjects were evaluated as part of a larger study in a battery of cognitive tests. For our study we focused on the complex figure task (CF) because previous research has shown that there is a correlation between performance on this test and hippocampal perfusion after exercise (Maass et al., 2015). Specifically, to assess spatial object recognition the Rey-Osterrieth Complex Figure (ROCF, Rey 1942) was used before exercise intervention and the Modified Taylor Complex Figure (MTCF, Strauss et al. 2006) at the end of the intervention as recommended for repeated measures of visual long term memory (Casarotti et al., 2014). The geometric pattern needed to be copied first and redrawn from memory after a delay of 3 minutes (early recall) and again after 30 minutes (late recall).

Blood collection

Blood samples were taken at rest in sitting position from the arm vein 1.5 hours before fitness assessment. The cooled (~4°C) blood samples were stored for 30 min before centrifugation at 2500 g for 15 minutes, supernatants were stored in 500µl aliquots at -80°C.

Missing data and outlier detection

CTSB and CTSL plasma levels were assayed by experimenters who were blinded with respect to group and fitness values. For 14 subjects, CTSB and CTSL plasma levels at one or more time points could not be used for statistical analyses due to debris in the blood sample. Two outliers

(exceeding 2.2 times the interquartile range from nearest quartile, Hoaglin et al., 1986) were excluded from the complex figure (CF) statistical analyses. For two subjects the dataset of the CF-test was missing or incomplete for either the pre- or post- test.

Tissue collection from mice

At the end of the scheduled experiments, mice were deeply anesthetized with isoflurane (Henry Schein Animal Health, OH) with O₂. Following cardiac puncture to collect blood, plasma samples were collected after centrifugation for 15 minutes at a minimum of 2500 g. Supernatants were stored at -80°C. The hippocampus, liver, spleen, white adipose tissue (WAT), soleus and gastrocnemius skeletal muscle were dissected and immediately frozen in liquid nitrogen and stored at -80 °C.

Identification of candidate myokine Cathepsin B

a. Myoblast cultures and preparation of conditioned media

Rat L6 skeletal myoblast cells (ATCC CRL-1458, VA) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, NY) supplemented with fetal bovine serum (FBS) to a final concentration of 10% at 37 C in 5% CO₂ in an incubator (Thermo Fisher Scientific, Waltham, MA). The cells were passaged every second day and confluency was maintained at less than 80% to prevent spontaneous differentiation. To induce differentiation, cells (4×10^4 cells/mL) were placed into DMEM supplemented with 2% FBS for 8 days. The media was changed every other day. Differentiation status was monitored daily under a microscope (Axiovert S100; Zeiss, Germany). L6 myotubes were washed four times with completely unsupplemented DMEM (no serum, phenol red, or antibiotics). Conditioned media were prepared by adding 12 mL of the unsupplemented DMEM, with 100 μM AICAR or distilled water (0.1% DMSO), to the myotubes and incubating them for 5 h in a 37°C CO₂ incubator. Conditioned media (CM) was collected

and centrifuged at 3000 rpm for 10 min (Eppendorf-5702, Hamburg, Germany), and then concentrated by a centrifugal filtering device (Millipore, Billerica, MA) by spinning at 3,500 x g for 45 min. This study used four experimental replicates for each condition consisting of 5×150 mm² culture dishes.

b. L6 Cells CM Silver Staining

The CM was run on a 4–12% Bis-Tris SDS-page gel (Novex, San Diego) and stained by silver staining method as described in company's manual (BioRad) with modification such as buffer incubation volume. The gel was fixed in 150 ml 50% methanol with 5% acetic acid for 20 minutes. Then the gel was sensitized with 0.02% Sodium Thiosulfate. After washing, the gel was reacted with silver for 20 min. Finally, the gel was developed with 150ml 2% sodium carbonate with 0.04% formalin (37%), and the reaction was stopped with 5% acetic acid water.

c. Mass spectrometry of L6 Cell proteins

In-gel protein digestion

Silver stained gel bands were digested following a published protocol with minor modifications (Shevchenko et al., 1996). Briefly, the bands were excised and destained in 100 µL of 2 mg/mL K₃Fe (CN)₆ solution for 10 min and washed 3 times with 400 µL water for 15 min. The destained gel was reduced by adding 100 µL of 20 mM dithiothreitol (DTT) for 60 min at 60°C and alkylated by 100 µL of 55 mM iodoacetamide (IAM) for 45 min in dark at room temperature. Supernatant was removed and the gel was washed multiple times with 100 µL of 100 mM ammonium bicarbonate buffer. The gel was dehydrated in 50 µL acetonitrile (ACN) for 10 min, followed by vacuum centrifugation to dryness, 50 µL of 0.02 µg/µL trypsin was added for overnight digestion at 37°C. Tryptic peptides were extracted with 50 µL of 5% ACN/0.1%

trifluoroacetic acid (TFA) twice and then 50 μL of 60% ACN/0.1% TFA twice. The extract was vacuum-centrifuged to dryness and resuspended in 15 μL of 5% ACN/0.1% FA.

LC-MS/MS mass spectrometry analysis

Peptide sample (5 μL) was introduced to a hybrid quadrupole-orbitrap mass spectrometer (Q Exactive™, Thermo Fisher Scientific, San Jose, CA) coupled to a nanoflow LC system (NanoAcquity; Waters Corporation, Milford, MA). A 100 μm i.d. x 20 mm pre-column was in-house packed with 200Å, 5 μm C18AQ particles (Michrom BioResources Inc., Auburn, CA). A 75 μm i.d. x 180 mm analytical column was pulled using a Sutter Instruments P-2000 CO₂ laser puller (Sutter Instrument Company, Novato, CA) and packed with 100 Å, 5 μm C18AQ particles. Mobile phase A was composed of 0.1% formic acid in water. Mobile phase B was 0.1% formic acid in acetonitrile. For each injection, an amount of 5 μL of sample was loaded on the pre-column at 4 $\mu\text{L}/\text{min}$ for 10 min, using a loading buffer of 5% acetonitrile and 0.1% formic acid. Peptide separation was performed at 250 nL/min flow rate in a 95 min gradient, in which mobile phase B started at 5%, increased to 35% at 60 min, 80% at 65 min, followed by a 5 min wash at 80% and a 25 min re-equilibration at 5%.

The eluted peptides were nano-electrospray ionized in positive ion mode. MS data was acquired in top-20 data-dependent acquisition experiment with 70K resolution for the full MS scan over an m/z range of 350-2000, 17.5K resolution for higher energy collisional dissociation (HCD) MS/MS scans. Precursor ions were selected from the full scan with an isolation width of 2 m/z for HCD fragmentation energy NCE = 35.

Data analysis

Tandem mass spectra were searched against a UniProtKB database of rat using Comet search engine v2014.01. Precursor mass tolerance was set at 10 ppm and a binning tolerance of

0.05 was used for fragment ions. Semi and full trypsin digestion after K or R (except when followed by P) with up to 2 missed cleavages, static carbamidomethylation and variable oxidation of methionine were defined as searching parameters. Peptide spectral matches were statistically validated using peptide-prophet and protein-prophet algorithms. Peptide and protein identifications were filtered at a minimum probability of 0.9 and 0.95, respectively (Database: <http://www.peptideatlas.org/> accession no. PASS00879).

In vitro analysis of Cathepsin B function

Adult hippocampal neural progenitor cells (aNPCs)

The aNPCs were prepared from the hippocampi of adult C57Bl/6J mice (kindly provided by Dr. Xinyu Zhao, Univ. Madison, WI). Cell proliferation and differentiation assays were performed as described previously (Ray and Gage, 2006) with minor modifications. Briefly, isolated aNPCs were grown in Neurobasal medium with B27 supplement (1:50), L-glutamine (1:100), EGF (20 ng/ml), bFGF (20 ng/ml) and heparin (20 ng/ml). The cells were fed every other day and sub-cultured every 6th day.

Annexin V apoptosis assay

For Annexin V apoptosis assay, aNPCs (4×10^5) were seeded into 12-well plates. After 24 hours cells were starved with Neurobasal medium without supplementary factors for 24 hours. Then cells were incubated with fresh medium containing PBS or recombinant Cathepsin B (R&D Systems) at 1, 10 or 100 ng/ml in a total volume of 1.0 ml per well for 24 h. The annexin V apoptosis assay was performed using an annexin V Alexa Fluor 647 conjugate (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Specifically, the cells were collected, washed once with PBS, and re-suspended in 100 μ l of annexin-binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl₂, pH 7.4), followed by incubation with 5 μ l of

annexin V conjugate solution and 5 μ l of 30 μ M propidium iodide (PI) for 15 min at room temperature. After the incubation period, 400 μ l of the annexin-binding buffer was added and mixed gently, and the samples were kept on ice. The samples were analyzed by flow cytometry (FACS Calibur; BD Coulter), and the data were analyzed using FACSDIVA software (BD Biosciences). For each sample, 10,000 gated cells were examined for their annexin V and PI staining, and the percentage of cells in early apoptosis was defined by measuring the high-annexin V- and low-PI-staining cell population, represented in the lower right quadrant of FACS analysis data. Minimum of two independent experiments was performed.

MTT assay

Cell viability was also quantified by using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) reagent (Sigma). MTT stock solution (5 mg/ml) was prepared in PBS and added to the culture media at a final concentration of 1 mg/ml. After 90 min incubation, the media was removed, and the chromogen in the cells was dissolved in DMSO containing 0.01 N NaOH. The absorbance at 540 nm was measured using a 96-well microplate spectrophotometer (Thermo Fisher Scientific).

Rat PC12 cells and transwell migration assay

PC12 cells were purchased from ATCC (Manassas, VA). PC12 cell suspension containing 2×10^5 cells in DMEM was applied to the upper well of a transwell chamber (24 wells, Corning), which was previously coated with laminin (5 μ g/ml, Sigma) on both sides. In the bottom well, DMEM with or without, FBS (100 ng/ml) and/or Cathepsin B (0.2–200 ng/ml) was applied to allow the cells to migrate across filters (8 μ m pore size). The transwell migration assay was performed in triplicate at 37°C for 5h. The cells in the upper well were mechanically removed, and cells in the lower well were fixed with 4% PFA/PBS for 10 min. After incubation with cresyl violet (Sigma)

at room temperature for 5 min, the cells were washed three times with PBS and examined by microscopy (Olympus BX51). Ten images were captured randomly (at 20X magnification) for each experiment and the number of cells in a 600×600 μm in each image was counted. Image J was used for counting as previously described (Koizumi and Nakajima, 2014). Relative percentages of the cell number to the average of the cell number in the control experiments (DMEM only in the bottom well) were calculated and plotted.

Semiquantitative RT-PCR analysis

Total RNA was extracted from L6, aNPC, PC12 cells and hippocampal, white adipose, muscle, spleen, liver and frontal cortex tissue using a total RNA extraction kit (Ribozol, Amresco) following manufacturer's manual. 5μg/10μl RNA was used for the cDNA synthesis. First-strand cDNA was synthesized by RT using oligo (dT) primers and SuperScript II reverse transcriptase (Invitrogen). cDNA was amplified for 15-25 cycles using primers as indicated below. For real-time RT-PCR, total RNA (100ng) was amplified using the PerfeCTa SYBR Green FastMix (Quanta Biosciences) and ECO PCR system (Illumina).

List of primers for qPCR

Gene		Primer sequence 5' - 3'	Species
Ctsb	F	GGCTACTCCACATCCTACAAG	Rattus
	R	ACACAGTAAAAGCACCCCTCC	
Ctsb	F	AGACCTGCTTACTTGCTGTG	Musculus
	R	GGAGGGATGGTGTATGGTAAG	
Hsp90	F	GAAGGACTACTGCACCAGAATG	Rattus
	R	CGTTCCACAAAGGCTGAGTTA	
Hsp90	F	GACCAAGGCTGACCTCATAAA	Musculus
	R	GACCAAGGCTGACCTCATAAA	
P11	F	GGGCTTCCAGAGCTTTCTATC	Rattus
	R	CTCGAAAGCTCCTCTGTCATT	

P11	F	CTTACGTTTCACAGGTTTGCAG	Musculus
	R	TTTTGTCCACAGCCAGAGG	
Dcx	F	AAGTGACCAACAAGGCTAT	Musculus
	R	TCATTGTGTTTTCCCGGA	
Bdnf	F	AAAATGCTCACACTCCA	Musculus
	R	GAACAAATGCTGGTCTT	

In vitro and in vivo analysis of CTSB

Western Blot analysis of cultured cells, muscle, and brain tissue

Cells were washed twice with cold PBS and lysed with RIPA buffer (Millipore) completed with Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology, Danvers, MA). For the tissue samples, frozen tissue was minced and sonicated with 1 second bursts for 15 seconds in ice. 300 μ L of lysis buffer was added per ~5mg of tissue. The lysates were centrifuged at 14,000 rpm for 15 min at 4 °C. For Western blot analysis, the supernatant fractions containing equal amounts of protein were subjected to SDS-PAGE and transferred to a NC membrane (Millipore). The membranes were blocked with 5% nonfat dried milk in PBS containing 0.1% Triton X-100 and then incubated with the appropriate primary antibodies at 4 °C overnight. [CTSB (CA10, Abcam), BDNF (N-20, Santa Cruz Biotechnology), DCX (C-18, Santa Cruz Biotechnology), P11 (aa.1-97, BD Biosciences), β -actin (Licor)]. Secondary antibodies linked to Infrared dye (680 Goat Anti-Mouse, 800 Anti-Goat, 800 anti-rabbit) were used at a dilution of 1:5,000. Specific signals were visualized by Odyssey (Amersham Biosciences).

ELISA assays

Cathepsin B quantification in plasma was performed using mouse, human Cathepsin B ELISA kits (Abcam, Cambridge, MA) and monkey Cathepsin B ELISA kits (Mybiosource, San Diego, CA) according to the manufacturer's specifications. Plasma Cathepsin L quantification was

carried out with human Cathepsin L ELISA kits (eBioscience, San Diego, CA). Briefly, frozen plasma samples were defrosted and then diluted 1:10-20 with Sample Diluent. Samples were loaded in duplicate on the plate and incubated according to manufacturer's protocol. Each assay included recombinant protein as a positive control. The plates were read using SpectraMax Plus 384 Microplate Reader (Molecular Devices, Sunnyvale, CA).

Intravenous injection of recombinant Cathepsin B

Anesthesia was induced by an intraperitoneal injection of Avertin (120 mg/kg). CTSB knockout mice were then injected with either the distilled water (vehicle, n=3) or recombinant CTSB protein (R&D Systems), 50 µg per mouse (n=3) intravenously (Wang et al., 1997; Yau et al., 2014). Fifteen minutes after injection, blood was collected by cardiac puncture and mice were perfused transcardially with 15ml of 0.9% saline. Thereafter, the brain was dissected out and lysed to perform ELISA assay as described above.

CTSB knockout mice

Male CTSB KO mice and WT littermates (6-7 weeks old) were housed individually in standard or running wheel cages with a standard day/night cycle: lights were switched on at 0600 and off at 1800. Following completion of behavioral testing at 15-16 weeks of age, animals were deeply anesthetized via inhalation of isoflurane and perfused transcardially with 0.9% saline (RT). The hippocampi and overlying cortices were dissected out of the left hemispheres and stored at -80°C for use in immunoblotting. The right hemispheres were stored in 4% paraformaldehyde (PFA) at 4°C for subsequent immunohistochemical experiments. After 96 h in PFA, tissue was equilibrated in 30% sucrose. Sequential coronal sections (40 µm) were taken through the hippocampus using a freezing microtome (HM450, Thermo Fisher Scientific) and stored in phosphate-buffered glycerol at -20°C.

Behavioral tests

Morris water maze

Mice were trained to find the hidden platform (15 cm dia) in the Morris water maze (1.4 m dia) (Morris, 1984). Mice were trained for 7 consecutive days with 4 trials per day. Starting points were changed daily for each trial. The animals were allowed a maximum of 60 s to locate the platform with a 15 s inter-trial interval. A 60 s probe trial was performed 24 h and 48 h after the last training session on day 7. Data collection was performed using the ANY-maze tracking system (Stoelting Co., Wood Dale, IL).

Rotarod

Motor function was evaluated with a five station accelerating rotarod stand-alone for mouse (Med Associates, St. Albans, VT). Two animals were tested at the same time. Each animal performed three consecutive five-min trials. The trials commenced with the mouse situated on the stationary rod for 10 s and the rod was then set in motion with an accelerating speed of 3 to 30 rpm. The latency to the first fall during the three consecutive five-min trials was recorded and averaged.

Open Field test

Mice were placed individually in an open field arena (27.3 × 27.3 cm, height 20.3 cm) housed within a sound-attenuating cubicle and permitted to move freely. Trials lasted 30 min. Animal motion and cumulative path length were automatically tracked via three 16-beam IR arrays and recorded by Activity Monitor software (Version 4.0, Med Associates, St. Albans, VT).

Elevated Plus Maze

Animals were individually placed in the center of an elevated plus maze (constructed on-site, made of white Plexiglas on a 60 cm high stand, each arm 30 cm x 5 cm, closed arms wall 16 cm

high, 5 cm x 5 cm center platform) and allowed to explore freely for 5 minutes. Time spent in the open and closed arms were recorded in semiautomatic fashion by video tracking software (ANY-maze, Stoelting Co., Wood Dale, IL).

Forced Swim test

The mice were placed in a cylindrical water tank (10 cm diameter and 25 cm depth) for 5 min. The water temperature was 24~26 °C. Mice were placed in the water tank for 5 min, and their behavior was videotaped. Analysis was performed by experimenters blinded to the group identity of the mice, and individually rated times for immobility, and mobility (swimming and climbing), and the average values of these ratings served as the final results. Immobility was defined as floating or no active movements made other than those necessary to keep the nose above water. Swimming was defined as active motions throughout the swim tank.

Sucrose preference

For this task mice were introduced to two dual bearing sipper tubes, one containing 2% sucrose and the other containing water. The mice were acclimatized to having two bottles for two days prior to the test, during these days the bottles contained water and 2% sucrose and these bottles were switched between days. On the third day the mice were given premeasured amounts of 4% sucrose and water. After 24 hours the amount consumed of each bottle was recorded and the mice went back to a single sipper tube with water.

Doublecortin immunohistochemistry and cell counts

A 1:12 series of coronal sections was stained for the presence of microtubule-associated protein doublecortin (DCX) (goat polyclonal, 1:10,000, Santa Cruz Biotechnology) for 1 h at room temperature and overnight at 4°C. After washing thoroughly, sections were reacted with biotinylated donkey anti-goat secondary antibody (1:250, Jackson ImmunoResearch).

Chromogen development with diaminobenzadine (DAB, Sigma) was carried out for 5 min. For analysis of DCX-positive cell number, two sections containing the DG were selected per mouse from comparable rostrocaudal (~1.46 mm and ~2.18 mm from bregma; Paxinos and Franklin, 2007) locations (van der Borgh et al., 2007). Quantification of DCX cells was carried out by manually counting and classifying each cell as type C or type D (Plumpe et al., 2006) at 40× magnification (Olympus BX51). For every mouse, the average number of cells/section was calculated.

Electrophysiology

Male CTSB KO (n=3) and WT littermates (n=3), between 4- to 6-weeks-old were deeply anesthetized with isoflurane and decapitated followed by quick transfer of the brain into dissection solution comprising (mM) 110 Choline-Cl, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 25 glucose, 1 CaCl₂, 7 MgCl₂, 0.6 Na⁺ pyruvate, 1.3 Na⁺ ascorbate, and 3 kynurenic acid.

Horizontal slices (300 μm thick) from dorsal to mid-dorsal hippocampus were obtained and transferred to incubation solution containing (mM): 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 2 CaCl₂, 2 MgCl₂, 20 glucose, 3 Na⁺ pyruvate and 1.3 Na⁺ ascorbate (equilibrated with 95% O₂ and 5% CO₂; pH 7.4, osmolarity- 310 mOsm). Slices were incubated at 34 °C for 10 min and stored at room temperature for at least 1 hour of recovery period. Patch clamp experiments were performed in artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 25 NaHCO₃, 2 CaCl₂, 1.3 MgCl₂ and 20 glucose, equilibrated with 95% O₂ and 5% CO₂; pH 7.4, osmolarity- 305 mOsm) in a submerged-type recording chamber (flow rate 2–2.5 mL/min at 30 °C).

For recording of miniature inhibitory postsynaptic currents (mIPSCs), pipettes were filled with (in mM): 130 CsCl, 10 HEPES, 0.1 EGTA, 5 Na-ATP, 0.5 Na-GTP, 10 Na-phosphocreatine

and 5 lidocaine N-ethyl bromide (QX-314) (pH 7.4, adjusted with CsOH). For recording intrinsic properties, pipettes were filled with (in mM): 130 K-gluconate, 7 KCl, 0.1 EGTA, 10 HEPES, 5 Mg-ATP, 0.5 Na-GTP and 10 Na-phosphocreatine (pH 7.4, adjusted with KOH). mIPSCs were recorded in presence of 0.5 μ M tetrodotoxin (TTX), 1 μ M CGP 55845 and 3 mM kynurenic acid to block sodium channels, GABA_B receptors and ionotropic glutamate receptors, respectively.

Mature GCs from the outer portion of the GC layer were identified using differential interference contrast video microscopy and recorded either in voltage clamp or current clamp mode. Whole-cell patch-clamp recordings were filtered at 2 kHz and digitized at 10 kHz and 20 KHz for voltage and current clamp recording, respectively using Multiclamp 700B, Digidata 1440A and pClamp 10.4 Software (Molecular Devices). Series resistance was typically within 10–30 M Ω .

mIPSC events were detected using amplitude threshold crossing and were analyzed in Igor Pro (Wave Metrics Inc., Lake Oswego, OR, USA). Threshold was typically kept at -5 pA and adjusted with respect to the baseline noise. Artifacts were screened through visual inspection and overlapping events (event frequency of ≥ 100 Hz) were discarded. Raw current traces of 300 s were analyzed for peak amplitude, frequency, rise time and decay time constant, which were averaged for each cell and used for further statistical analysis. A single exponential function was fitted to decay times of individual events to evaluate the decay time constant.

Input resistance was determined from the slope of the linear regression fit through the V–I plot constructed by plotting the amplitude of the steady-state voltages (V) against the corresponding step current injections (I) from -10 to +15 pA of 500-ms duration with 5 pA increment. Membrane time constant was estimated by fitting a single exponential function to a 500 ms voltage trace in response to -10 to +10 pA current injection in steps of 5 pA and

averaging them. Membrane capacitance was calculated as a ratio of membrane time constant and input resistance.

Statistics

All statistical analyses for the mouse and monkey studies were performed using Statview. Unpaired student's t tests were used for two-group comparisons. One-way ANOVAs were used for ELISA, qPCR, Western blotting, FACS analysis. Two-way ANOVAs were used for the CTSB KO studies with Housing (sedentary or exercise) x Genotype (WT or KO) as independent variables. Two-way ANOVAs were used for the P11 siRNA experiments in PC12 cells with treatment (vehicle or recombinant CTSB) x transfected cells (scramble or P11 siRNA) as independent variables. qPCR analysis, ELISA experiments, western blotting intensity analysis and behavioral analyses were followed by Bonferroni's post hoc tests. For the human studies, statistics were calculated using SPSS (Version 21.0.0.2). One-way ANOVA with repeated measures over time was used to analyze CTSB, CTSL, VO₂ and lactate levels. Percentage changes of fitness, CTSB in plasma and differences of memory performance over time points were correlated using bivariate Pearson correlation with pairwise deletion criteria.

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