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Untranslated regions of brain-derived neurotrophic factor mRNA control its translatability and subcellular localization

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Brain-derived neurotrophic factor (BDNF) promotes neuronal survival and growth during development. In the adult nervous system, BDNF is important for synaptic function in several biological processes such as memory formation and food intake. In addition, BDNF has been implicated in development and maintenance of the cardiovascular system. The *Bdnf* gene comprises several alternative untranslated 5′ exons and two variants of 3′ UTRs. The effects of these entire alternative UTRs on translatability have not been established. Using reporter and translating ribosome affinity purification analyses, we show that prevalent *Bdnf* 5′ UTRs, but not 3′ UTRs, exert a repressive effect on translation. However, contrary to previous reports, we do not detect a significant effect of neuronal activity on BDNF translation. *In vivo* analysis *via* knock-in conditional replacement of *Bdnf* 3′ UTR by bovine growth hormone 3′ UTR reveals that *Bdnf* 3′ UTR is required for efficient *Bdnf* mRNA and BDNF protein production in the brain, but acts in an inhibitory manner in lung and heart. Finally, we show that *Bdnf* mRNA is enriched in rat brain synaptoneurosomes, with higher enrichment detected for exon I–containing transcripts. In conclusion, these results uncover two novel aspects in understanding the function of *Bdnf* UTRs. First, the long *Bdnf* 3′ UTR does not repress BDNF expression in the brain. Second, exon I–derived 5′ UTR has a distinct role in subcellular targeting of *Bdnf* mRNA.

Brain-derived neurotrophic factor (BDNF) (1), a member of the neurotrophin family, supports neuronal survival in mammalian development (2, 3). In the adult brain, BDNF modulates synaptic activity and plays central roles in memory formation and maintenance (4, 5). Dysregulated BDNF has been implicated in depression and other central nervous system disorders (6). BDNF is a key regulator of food intake and body weight (7), and *Bdnf* haploinsufficiency has been causally linked to severe hyperphagia in WAGR syndrome (Wilms tumor, aniridia, genitourinary anomalies, and mental retardation) patients (8). In addition to the nervous system, *Bdnf* mRNA is highly expressed in the heart and lung (9). BDNF has been shown to regulate heart development (10) and modulate adult cardiac contractility (11, 12). In the lung, BDNF-TrkB signaling promotes regeneration of alveolar epithelial cells after injury (13). Owing to its pleiotropic functions, molecular mechanisms governing BDNF expression are of high clinical interest.

The *Bdnf* gene has a complex structure with several promoters preceding alternative 5′ exons that are spliced with a common 3′ exon encoding the BDNF protein (14, 15) (Fig. S1*A*). The multipromoter arrangement presents a convenient way to fine-tune BDNF expression in a cell type–specific manner and as a response to neuronal activity and other stimuli (16). Promoter IV–driven BDNF has a critical role in the development of cortical inhibition (17, 18) and its disruption in mice causes defects in sensory processing (19). Disruption of promoter I– and II–driven BDNF in mice has been shown to induce aggressive behavior with accompanying expression changes in serotonin signaling pathways (20). In addition to allowing transcriptional control over BDNF expression, the multipromoter arrangement generates *Bdnf* transcripts with alternative 5′ UTRs. Compared to alternative promoter use, the roles of alternative *Bdnf* UTRs are much less clearly understood.

By forming secondary structures and interacting with different RNA-binding proteins, 5′ UTRs can affect several aspects in the fate of an mRNA including its subcellular localization and translation efficiency (21). The effect of alternative *Bdnf* 5′ UTRs on subcellular localization in neurons has been thoroughly studied, concluding that exon II– and VI–containing UTRs promote dendritic targeting (22–24). The effect of *Bdnf* UTRs on reporter translatability has been studied in SH-SY5Y cells, where the authors identified a differential response pattern upon stimulation of cells with an array of neurotransmitters (25). Previously, we have shown that exon I– and exon IV–containing mRNAs repress BDNF

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protein translation in primary cortical neurons (26). However, a comparative analysis of all 5′ UTRs in primary neurons is currently missing. In this study, we have for the first time systematically analyzed the effects of all alternative rat *Bdnf* UTRs on mRNA translatability in rat primary cortical neurons and conclude that commonly used 5′ UTRs repress translation to a similar extent.

Use of alternative polyadenylation sites generates *Bdnf* mRNAs with 3′ UTRs of two different lengths (9, 27). It has been reported that *Bdnf* mRNA containing the long, but not short 3′UTR, is targeted to hippocampal dendrites, where it undergoes local translation (28). This finding, however, has been challenged by studies failing to detect *Bdnf* mRNA (29) or protein (30) in dendrites. *Bdnf* long 3′ UTR has been reported to act as a translational suppressor at rest, getting derepressed by neuronal activity and allowing BDNF synthesis to proceed (31). *Bdnf* 3′ UTRs contain multiple miRNA-binding sites and several of these have been experimentally shown to modulate *Bdnf* translation (32–34). Additionally, global perturbation of miRNA processing by CamK2a-Cre conditional knockout of Dicer has been shown to increase BDNF protein levels (35). Truncation of *Bdnf* long 3′ UTR in mice displayed reduced *Bdnf* mRNA in the hypothalamus and developed severe hyperphagic obesity (36). Taken together, it has remained inconclusive whether *Bdnf* 3′ UTRs have a repressive or permissive effect on its expression. In this study, we use three different *in vitro* approaches—luciferase assay, translating ribosome affinity purification(TRAP), and *de novo* protein synthesis assay—to show that the long *Bdnf* 3′ UTR does not have repressive effects on protein synthesis. Moreover, by *in vivo* experiments, using a novel conditional 3′UTR replacement approach (37, 38), we demonstrate reduced BDNF expression in the brain, indicating that the *Bdnf* 3′ UTR indeed does not have a repressive effect in this tissue. In contrast, 3′ UTR replacement leads to higher BDNF levels in heart and lungs, suggesting differential 3′UTR–targeted mechanisms of posttranscriptional regulation of *Bdnf* expression in neuronal and nonneuronal tissues.

Finally, we studied subcellular localization of *Bdnf* mRNA in synaptoneurosomes (SNs). We find that all major *Bdnf* mRNA isoforms (including long 3′ UTR–containing isoforms) are enriched in SNs, at levels comparable to *Camk2a*, a known synaptically enriched mRNA. Among *Bdnf* mRNAs, the highest synaptoneurosomal enrichment was detected for exon I–containing transcripts. Taken together, we demonstrate that in neurons, *Bdnf* 5′UTRs, but not 3′ UTRs, repress translation and show a previously unknown subcellular enrichment for exon I–containing transcripts.

Results

Bdnf *5′ but not 3′ UTRs repress protein synthesis in primary neurons*

To investigate the effect of *Bdnf* UTRs on mRNA translatability in primary neurons, we used dual luciferase reporter assay. Cortical neurons were transfected with firefly luciferase constructs where either *Bdnf* or control 5′ and 3′ UTRs were cloned around the luc2P ORF (Figs. 1*A* and S1, *A* and *B*). For *Bdnf* 5′ UTRs, we included sequence variants arising from previously determined alternative transcriptional start sites (27, 39) (Fig. S1*B*). Seventeen out of the 21 alternative 5′ UTR variants tested displayed a repressive effect on luciferase activity (Fig. S1*C*), including all most commonly used variants arising from exons I, II(c), IV, and VI (Fig. 1*B*) (27, 39), and none of the 5′ UTRs showed increased translation compared to the control 5′ UTR (Figs. 1*B* and S1*C*). The strongest repressive effect was observed for the longest exon VI 5′ UTR (VI-1, > 100-fold repression), exon VIII variants (∼6-fold), exon I variants (∼3-fold), and the main variant of exon IIc (∼3-fold) (Fig. S1*C*). The hyper-repressive effect of VI-1 is in line with previous results (25) and arises from using an upstream ATG that generates an ORF out of frame with the luciferase ORF (Fig. S1, *E* and *F*). When analyzing the effect of 3′ UTRs, we found that the long, but not short 3′ UTR isoform, caused a decrease in luciferase signal (Fig. 1*B*).

As alternative UTRs could also affect RNA stability (40), we measured the levels of reporter RNA by quantitative reverse transcription PCR and recalculated the translatability scores in Figures 1*B* and S1*C* as normalized by RNA levels (Figs. 1*C* and S1*D*). After RNA amounts were accounted for, all 5′ UTRs still exerted a repressive effect on luciferase reporter translation, with a more pronounced repression detected for exon IIc UTR (the longest alternative splicing variant of exon II). Overall, normalization with mRNA levels did not change the outcome of luciferase assay results for the analysis of 5′ UTRs. In contrast, normalization completely changed the interpretation for 3′ UTRs: long and short *Bdnf* 3′ UTR variants both displayed similar translatability after normalization (Fig. 1*C*), suggesting that in cortical neurons, the long 3′ UTR does not have a direct repressive effect on protein synthesis but decreases RNA stability.

Next, we used TRAP (41) to test translatability of endogenous *Bdnf* mRNA. Primary hippocampal neurons were transduced with adeno-associated viruses (AAVs) carrying Rpl10a-EGFP driven by the neuron-specific human *Synapsin* I promoter (Syn) (Fig. 1*D*). A ratio between immunoprecipitated ribosome-associated RNA and total RNA used for input samples was used to calculate a relative translatability index for the comparison of different RNAs. RT-qPCR analysis showed that transcripts containing *Bdnf* exon IIc were significantly less associated with translating ribosomes than total *Bdnf* mRNA (detected with primers against the coding sequence), in agreement with our dual luciferase assay results. Ribosome recruitment of exon I, IV, VI, or *Bdnf* long 3′ UTR–containing RNAs was not significantly different from that of total *Bdnf* mRNA (Fig. 1*E*). As a reference, we quantified *Camk2a*, a known dendritically localized mRNA (42) that is basally repressive and undergoes neuronal activity–dependent translation by a cytoplasmic polyadenylation-dependent mechanism (43). *Camk2a* showed significantly lower translatability than *Bdnf* mRNA (Fig. 1*E*).

Finally, we labeled newly synthesized proteins as described in (44) to study the effect of the long *Bdnf* 3′ UTR on *de novo* synthesis of the firefly luciferase reporter protein. To this end, we transfected HEK293 cells with the control and *Bdnf* 3′ UTR

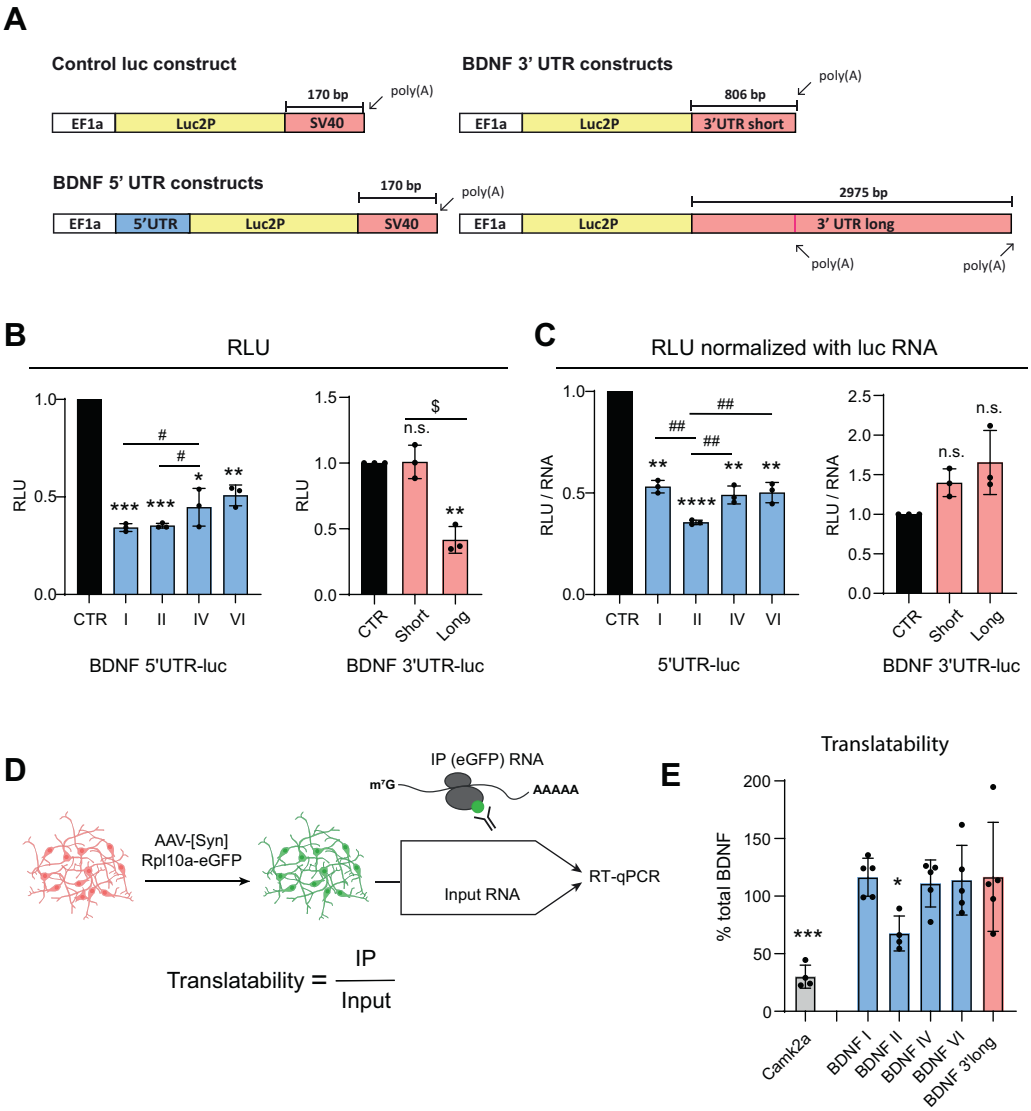

Figure 1. The effect of *Bdnf* UTRs on reporter translatability and ribosome association. *A*, schematic of firefly-luciferase reporter constructs used for studying the effect of *Bdnf* untranslated regions on mRNA translatability. 3′ untranslated regions are indicated in *red*, poly(A) denotes alternative polyadenylation sites. *B*, dual luciferase reporter assay in rat primary cortical neurons at 6 days *in vitro* (DIV) and 24h after reporter transfection. Cells were simultaneously transfected with indicated firefly luciferase constructs and Renilla luciferase for normalization, both under the EF1a promoter. Firefly luciferase constructs carry either a minimal 5′ UTR and *SV40* 3′ UTR (control, CTR), one of the *Bdnf* 5′ UTRs inserted in front of the start codon of luciferase ORF (*blue bars*) or 3′ UTR replaced by either short or long variant of the *Bdnf* 3′ UTR (*red bars*). RLU relative to the "CTR" sample taken as 1. *$p < 0.05$ **$p < 0.01$, ***$p < 0.001$ (one sample *t* test comparing the means with a control mean of 1); # $p < 0.05$ (one-way ANOVA with Tukey's post hoc test); $ $p < 0.05$ (unpaired two-tailed *t* test). Error bars – SD; n = 3. *C*, luciferase signal (RLU) normalized to luciferase mRNA (see Experimental procedures for details). **$p < 0.01$, ****$p < 0.0001$ (one sample *t* test comparing the means with a control mean of 1.0 for CTR); ## $p < 0.01$ (one-way ANOVA with Tukey's post hoc test); Error bars – SD; n = 3. *D*, translating ribosome affinity purification experiment (TRAP). Rat hippocampal neurons were prepared from E21 rat embryos and transduced with AAV1/2 vectors expressing Rpl10a-EGFP from a neuron-specific *Synapsin* I promoter ([Syn]). Immunoprecipitation of translating ribosomes was performed at 2 weeks of culture to capture ribosome-associated mRNA. The translatability metric was calculated as a ratio of ribosome-bound (IP) and total RNA (input) in RT-qPCR analysis. *E*, translatability of *Camk2a* mRNA and *Bdnf* alternative transcripts as compared to total *Bdnf* mRNA taken as 100%. Note that *Bdnf* mRNAs carrying the short 3′ UTR cannot be selectively measured by PCR as this sequence is also contained in the long 3′UTR. Error bars – SD; n = 4. *$p < 0.05$, ***$p < 0.001$ (one sample *t* test comparing the means of with total *Bdnf* levels (100%)). BDNF, brain-derived neurotrophic factor; AAV, adeno-associated virus; EF1α, elongation factor 1 alpha promoter; Luc2P, destabilized firefly luciferase ORF; 5′ UTR, one of rat *Bdnf* 5′ untranslated regions; RLU, relative luciferase units; IP, immunoprecipitation.

luciferase constructs used for luciferase assays above (see Fig. 1*A*), labeled newly synthesized proteins with O-propargyl puromycin (OPP), click-conjugated labeled proteins with biotin azide, and isolated biotinylated proteins by streptavidin pulldown as described in (44) (Fig. 2*A*). Western blot analysis of *de novo*–synthesized firefly luciferase protein (pulldown) and steady state luciferase protein (input) showed a higher pulldown to input ratio for long 3′ UTR constructs than the short 3′ UTR and control (SV40) 3′ UTR constructs, suggesting a facilitating effect on translation by the long *Bdnf* 3′ UTR (Fig. 2, *B–D*).

Decreased brain and increased non-neuronal Bdnf *in 3′ UTR replacement knock-in mice*

Focusing on the regulation of *Bdnf* by the 3′ UTR, we next studied BDNF expression levels in a conditional 3′ UTR

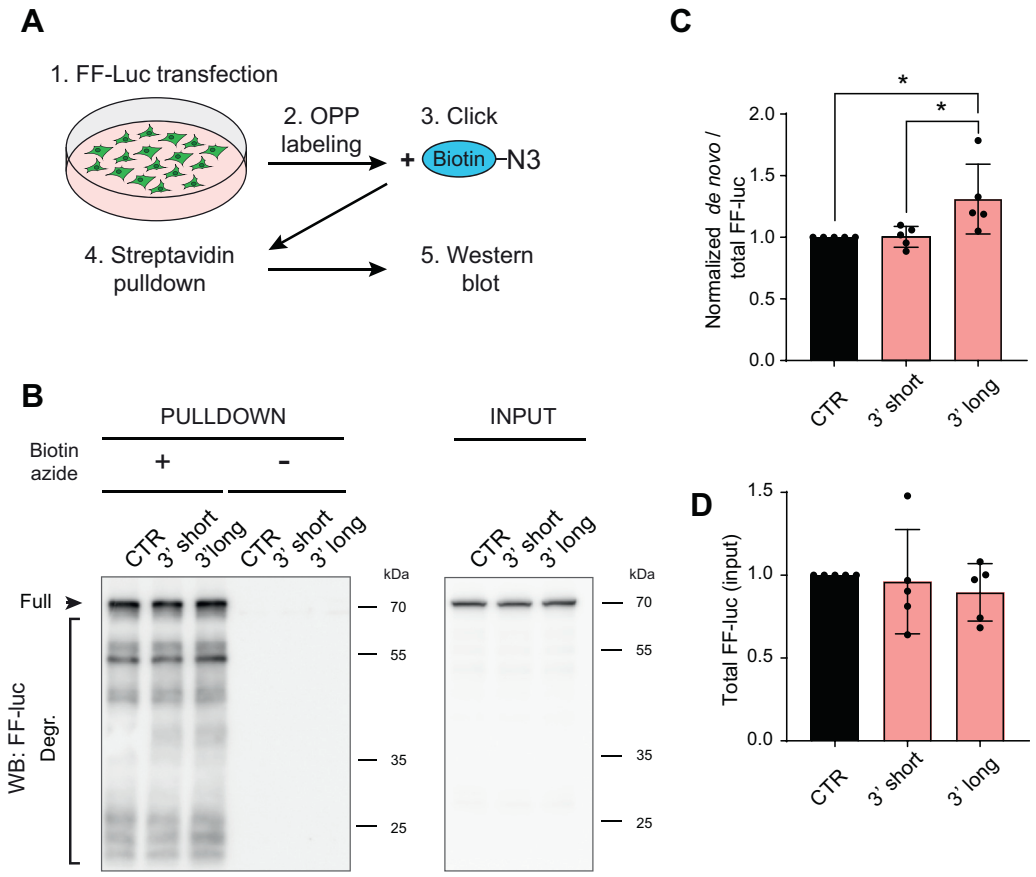

Figure 2. *Bdnf* long 3′ UTR facilitates *de novo* synthesis of the firefly luciferase reporter protein. *A*, HEK293 cells were transfected with luciferase reporter constructs carrying either the control *SV40* 3′UTR, short or long *Bdnf* 3′UTRs (see Fig. 1*A*). Cells were pulse labeled with 20 μM OPP for 1h, and cell lysates were conjugated with biotin azide (in control samples, biotin azide was omitted from the reaction). *De novo*–synthesized protein was isolated by streptavidin pulldown and analyzed by Western blot using anti-firefly luciferase antibody. *B*, example Western blot from the experiment. Input samples (approximately 1% of protein that was used for pulldown) were analyzed for reference. Full-length luciferase protein is indicated with an arrowhead and luciferase degradation products in pulldown samples with "Degr." The degradation products of variable sizes in pull-down samples likely arise due to the PEST degron attached to the C-terminus of the protein. *C*, quantification of luciferase signal (full size + degradation products) in pulldown samples, normalized to input levels. Shown are means ± SD, n = 5 independent replicates. *D*, quantification of luciferase signal in input samples, normalized to Coomassie staining of the membrane. Means ± SD, n = 5 independent replicates. *$p < 0.05$ (one-way ANOVA with Tukey's post hoc test). BDNF, brain-derived neurotrophic factor; OPP, O-propargyl puromycin.

replacement knock-in mouse model (Fig. 3*A*). To this end, we generated mice with targeted insertion of inverted floxed bovine growth hormone (*bGH*) 3′ UTR between the *Bdnf* stop codon and its native 3′ UTR (Figs. 3*A* and S2*A*). We chose the *bGH* 3′ UTR since, to the best of our knowledge, it ensures the highest levels of transgene expression by promoting mRNA stability (38). Cre-dependent inversion of the the *bGH* cassette using FLEX sequence (45) results in the transcription of *Bdnf* mRNAs containing the *bGH* 3′ UTR instead of the endogenous 3′ UTR sequences (Fig. 3*A*). Mice carrying the conditional *Bdnf* 3′ UTR replacement to bGH 3′UTR (*Bdnf* c3′ R) allele were crossed with a deleter-Cre line (PGK-Cre; (46)), which results in ubiquitous recombination detectable in tail DNA (data not shown). Offspring was used to compare *Bdnf* expression in littermates. Western blot analysis of brain tissue in mice revealed a broad decrease in BDNF protein across different brain regions in an allele dose-dependent manner in mice where native *Bdnf* 3′ UTR was replaced with the *bGH* 3′ UTR (Fig. 3, *B* and *C*). Strikingly, we observed the opposite effect in heart and lung (Fig. 3, *B* and *C*), two non-neuronal tissues expressing BDNF (9, 27). *Bdnf* mRNA analysis by RT-qPCR showed similarly decreased levels in the brain regions of homozygous *Bdnf* c3′ R mice (Fig. S2*B*), suggesting that decreased mRNA stability contributes to reduced BDNF protein levels.

No evidence for activity-dependent translation of BDNF mRNAs with different UTRs

Neuronal activity–dependent activation of BDNF translation has been reported using rat hippocampal cultures and the KCl depolarization model (47). That study also showed KCl depolarization-induced dendritic localization and translation of a GFP reporter RNA containing both rat *Bdnf* exon VI 5′UTR and long 3′UTR (47). Therefore, we were interested in assessing whether neuronal activity can modulate the effect on translatability in cultured neurons by any of the alternative *Bdnf* UTRs. To test this, we transfected rat cortical or hippocampal neurons with UTR-luc constructs (same as in Fig. 1, *A* and *B*) and stimulated the cells with KCl depolarization

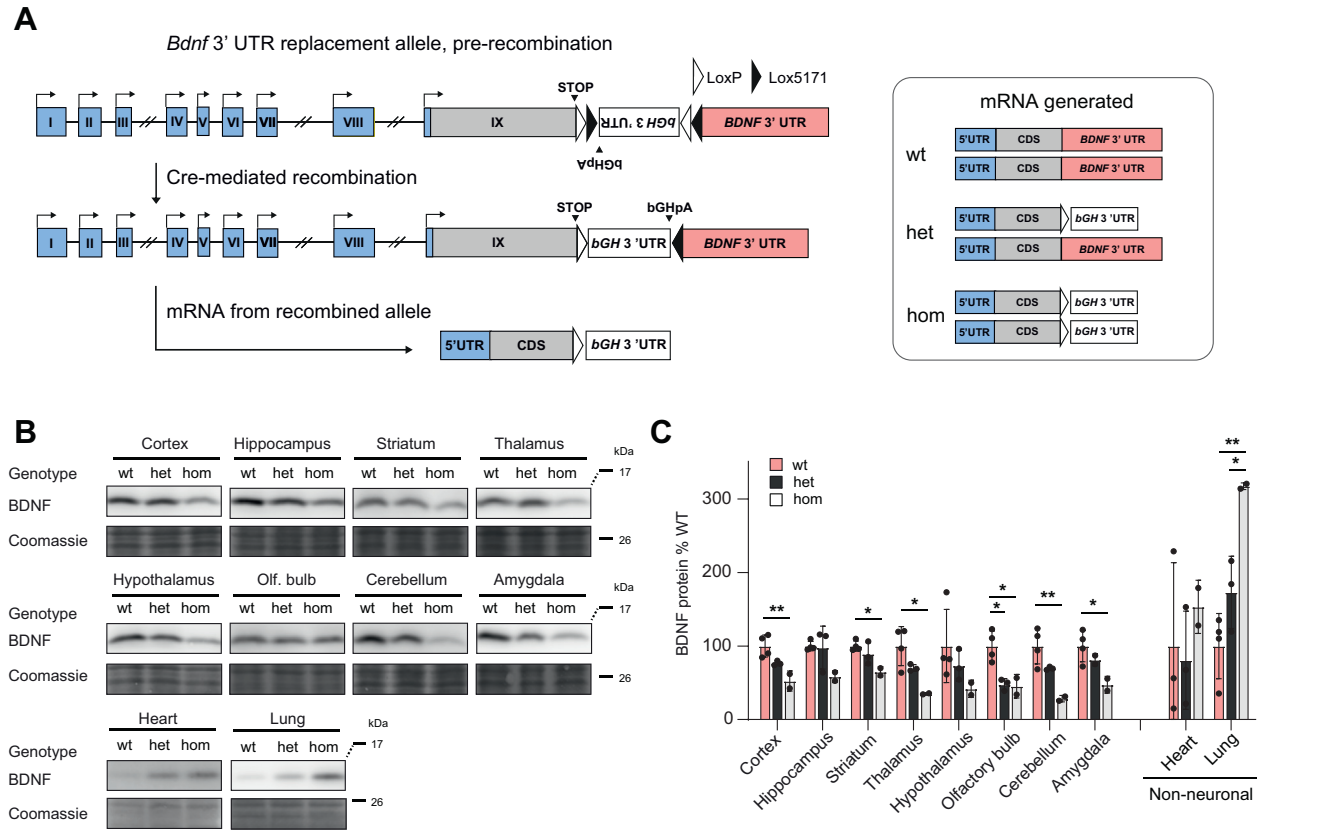

Figure 3. *Bdnf* 3′ UTR replacement by *bGH* 3′UTR in mice. *A*, schematic of the conditional *Bdnf* 3′ UTR replacement (c3′ R) allele and expression of the bovine growth hormone (*bGH*) 3′ UTR upon Cre-mediated recombination. *Right*: schematic of mRNAs generated from the *Bdnf* locus in mice analyzed in (*B* and *C*). *B*, Western blot analysis of mature BDNF protein in total lysates of brain regions, and immunoprecipitated (IP) fractions of heart and lung tissue from wt, heterozygous (het), or homozygous (hom) c3′ R mice. Coomassie staining of membranes was used to test for equal loading and for normalization of chemiluminescence signal. For heart and lung, IP flow-through samples were run separately and stained with Coomassie. Example BDNF Western blots and Coomassie-stained membrane cutouts for the corresponding lanes are shown. *C*, quantification of (B). Coomassie-normalized BDNF protein levels are expressed as per cent relative to the mean value of WT group. n = 2 to 4 animals per genotype. Shown are means ± SD; *$p < 0.05$, **$p < 0.01$ (one-way ANOVA with Tukey's test). BDNF, brain-derived neurotrophic factor.

(55 mM) for 15 min or 3 h before measuring luciferase activity (Fig. 4, *A* and *B*). The time points were selected based on reported KCl-induced BDNF protein elevation and induced luciferase from *Bdnf* VI 5′ UTR/3′ UTR long reporter (47). This analysis revealed a modest activity-dependent induction of luciferase translation from *Bdnf* exon-II–containing mRNA in cortical neurons at 3h but not at 15 min of depolarization. In hippocampal neurons, we detected a small increase in translation from reporters containing exon VI UTR, short 3′ UTR and exon VI UTR in combination with 3′ UTR long. Taken together, no single UTR nor the combination of 5′ UTR VI and or 3′ UTR long showed robust and reproducible activity-dependent regulation in both culture systems analyzed in our study.

Next, we tested the regulation of BDNF translation at the endogenous protein level in cultured neurons. We treated matured cortical neurons (DIV 7) with 55 mM KCl or with 100 μM glutamate for 5 to 15 min and quantified BDNF protein by immunoblotting (Fig. 4, *C* and *D*). We focused on these short treatment periods for two main reasons: first, *Bdnf* transcription is strongly regulated by neuronal depolarization (16) and increases in protein levels at time points longer than 30 min would also reflect transcriptional responses. Second, the effects of neuronal activity on global translation (48–50) or on translation of individual proteins such as BDNF (47, 51), CamKIIa (50), Arc (52), Wnt5a (53), and MMP9 (54) have been demonstrated to occur in the time scale of 3 to 30 min. Here, we did not observe stimulus-dependent increase in mature BDNF protein levels (proBDNF was undetectable in these experiments, data not shown) with any of the used treatments, suggesting that neuronal activity does not induce BDNF translation at these time points (Fig. 4, *C* and *D*). However, both mature and proBDNF proteins are readily induced at 3 h of KCl depolarization, most likely completely driven by activity-dependent BDNF transcription (a representative blot is shown in Fig. 4*E*).

Bdnf *mRNAs are enriched in SNs*

Finally, we assessed if there is differential abundance of certain *Bdnf* transcripts in brain SNs. mRNA targeting to distal compartments and local translation is used by neurons for timely adjustments of their local protein repertoires and to overcome the large distances these cells span (55–58). mRNA targeting "zip code" sequences have often been associated with 3′ UTRs (59, 60), but 5′UTR-embedded localization motifs

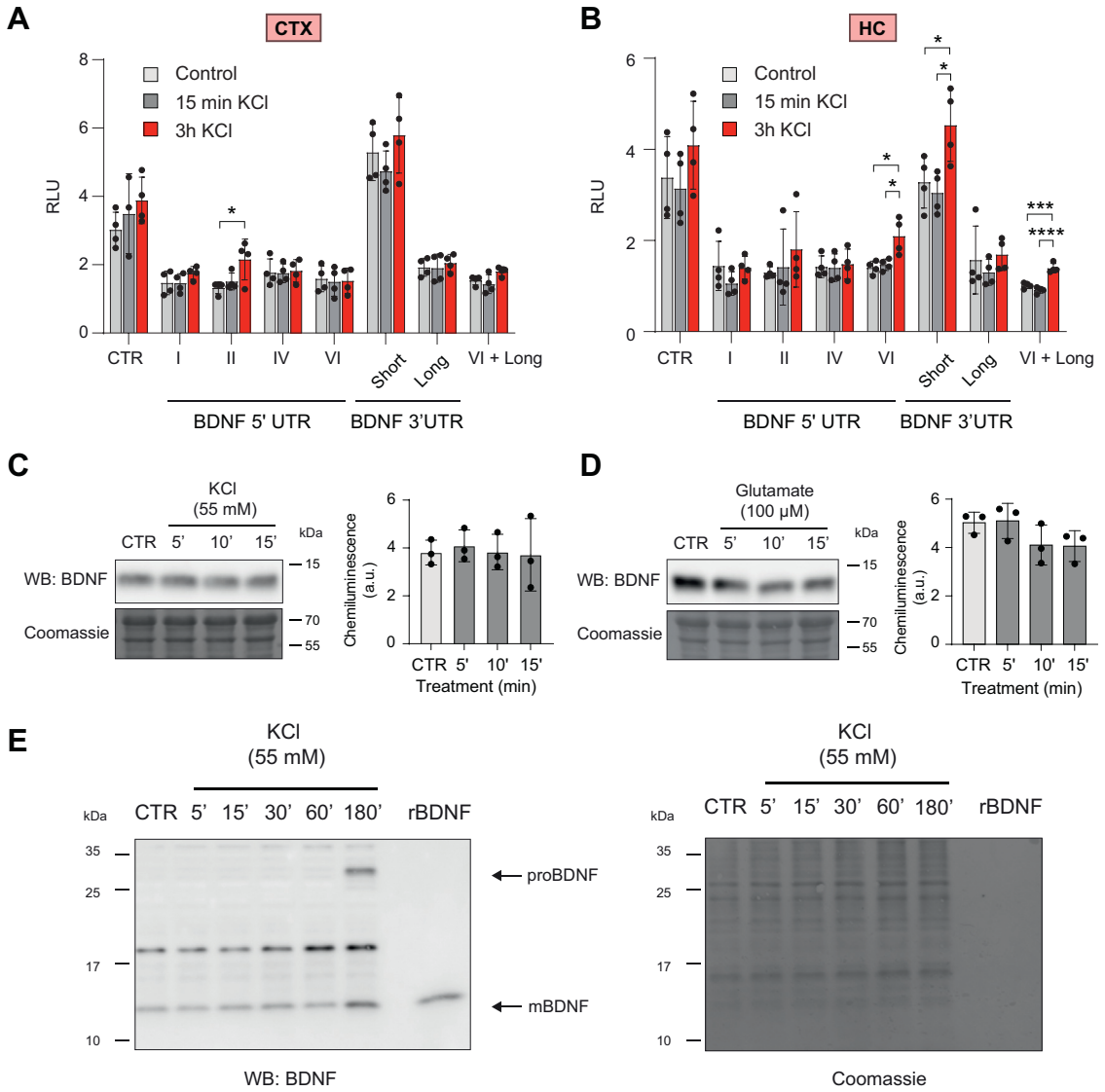

Figure 4. Activity-dependent modulation of *Bdnf* translatability. *A* and *B*, dual luciferase reporter assay in rat primary cortical (*A*) or hippocampal (*B*) neurons, cultured for 5 days *in vitro* and transfected as in Figure 1. Twenty four hours after transfection, neurons were treated with 55 mM KCl for 15 min or 3h, or left untreated before measuring luciferase activity. Error bars – SD; n = 4. *$p < 0.05$, ***$p < 0.001$. ****$p < 0.0001$ (one-way ANOVA with Tukey's post hoc test). *C* and *D*, Western blot analysis of BDNF in rat cortical neurons cultured for 7 DIV and treated for 5, 10, or 15 min with 55 mM KCl (*C*) or 100 µM glutamate (*D*), or left untreated (CTR). Representative BDNF blots and cutouts from corresponding Coomassie-stained membranes are shown to verify equal loading. Graphs on the *right*: BDNF levels were quantified from four independent experiments and expressed as mean chemiluminescence values (Error bars – SD). No significant effect of treatment on BDNF levels was detected at any tested time point for any of the treatments (one-way ANOVA). *E*, Western blot analysis of BDNF in rat cortical neurons cultured for 7 DIV and treated for 5 to 180 min with 55 mM KCl or left untreated (CTR). Coomassie staining of the membrane on the left is shown for reference, showing equal loading of protein. Bands corresponding to proBDNF and mature BDNF (mBDNF) are indicated. BDNF, brain-derived neurotrophic factor; CTR, control firefly luciferase vector; VI+Long, vector carrying a combination of exon VI 5′ UTR and long 3′ UTR; RLU, relative luciferase units; rBDNF, lane loaded with 100 pg of recombinant BDNF.

have been described as well (61, 62). We were intrigued to study the abundance of *Bdnf* transcripts bearing alternative 5′ and 3′ UTR sequences in SNs, a subcellular fractionation preparation containing membrane-sealed presynaptic and postsynaptic compartments and fine perisynaptic astrocyte processes. We prepared SNs from the brains of adult rat cortical and hippocampal tissues as per De Rubeis and Bagni (63) and tested the purity of fractions by immunoblotting with subcellular compartment markers (Fig. 5*A*). This analysis showed that synaptoneurosomal fractions from both tissues were depleted of cell nuclei (probed with acetylated histone H3) and enriched in an abundant synaptic protein CamKIIa (64), presynaptic proteins Synapsin I (65) and BDNF (30), and postsynaptic protein PSD95 (66); GAPDH was used as a loading control (Fig. 5*A*). Next, we isolated RNA from the synaptosomal fraction and profiled the enrichment of total *Bdnf*, *Bdnf* mRNAs with alternative 5′ UTRs, and *Bdnf* mRNA containing 3′ UTR long (Fig. 5, *B* and *C*). For reference, we measured the enrichment of a benchmark dendritic transcript *Camk2a* (42, 67), ubiquitously localized transcript *Tuba1* (67), and a nuclear-retained noncoding RNA *Malat1* (68). First, we found that pan-*Bdnf* transcripts (*Bdnf* coding) as well as all

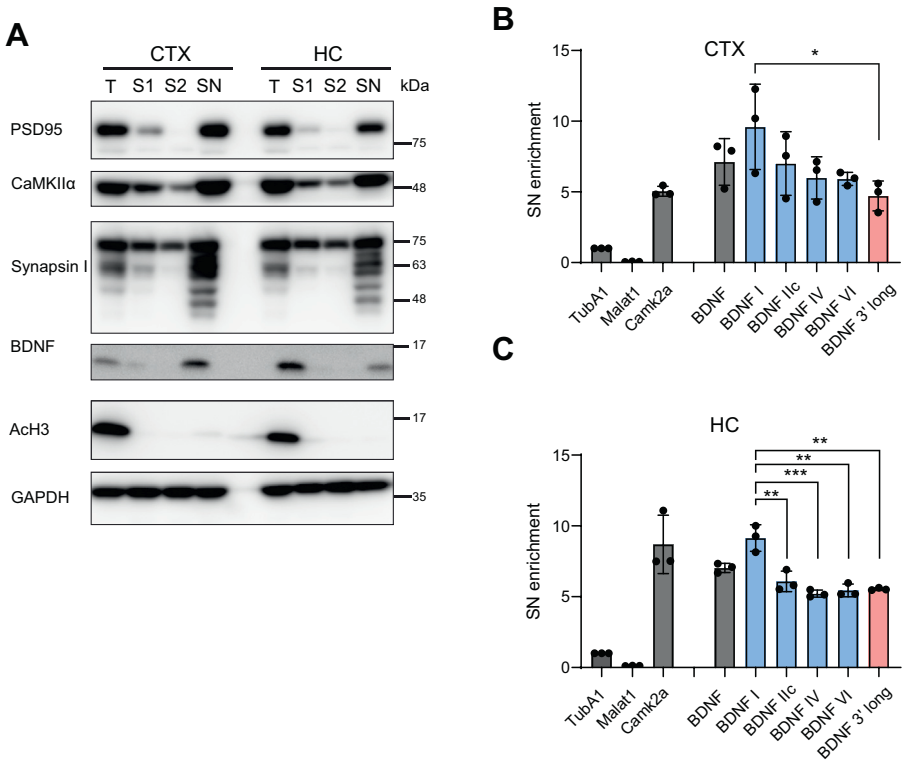

Figure 5. Localization of alternative *Bdnf* transcripts into synaptoneurosomes prepared from adult rat cerebral cortex and hippocampus. *A*, Western blot analysis of synaptoneurosome (SN) purification fractions, with antibodies for synaptic proteins (PSD95, CamKIIα, Synapsin I, and BDNF), nuclear marker acetylated histone H3 (AcH3), and loading control GAPDH. *B*, enrichment of indicated mRNAs in cortical SNs relative cytosolic levels (fraction S2), with Tuba1 levels taken as 1. n = 3, $*p < 0.05$, ANOVA with Tukey's post hoc test; error bars – SD. *C*, enrichment of indicated mRNAs in hippocampal SNs, normalized to levels in the cytosolic fraction (S1). n = 3, $**p < 0.01$, $***p < 0.001$, ANOVA with Tukey's post hoc test; error bars – SD. BDNF, brain-derived neurotrophic factor; T, total unfractionated lysate; S1, supernatant depleted of nuclei, containing cytoplasmic fraction and synaptoneurosomes; S2, cytoplasm from the next supernatant de-enriched for synaptoneurosomes; SN, purified synaptoneurosome fraction.

Bdnf transcript variants were similarly enriched in SNs when compared to *Camk2a* mRNA. We found that, exon I–containing transcripts showed higher enrichment than exon II–, IV–, and VI–containing *Bdnf* mRNAs.

Discussion

In this study, we have examined the effect of alternative *Bdnf* UTRs on translational efficiency and mRNA localization in neurons *in vitro* and *in vivo*. We found that in primary cortical neurons, most 5′ UTR variants, including all major 5′ UTRs (I, IIc, IV, and VI) have a repressive effect on translatability. Among these, exon IIc (the longest alternative splicing variant of exon II) UTR showed the strongest repressive effect, whereas shorter exon II variants IIa and IIb were found to be less repressive. These results are in good agreement with a similar analysis performed in neuroblastoma SH-SY5Y cells (25). The latter study showed reduced luciferase activity for three out of nine *Bdnf* alternative 5′ UTRs tested, including strong repression by 5′ UTRs of exons I, IIc, IV, VI, and VIII. In agreement with our results, Vaghi *et al.* (25) did not observe a strong repression for exons III and V 5′ UTRs in neuroblastoma SH-SY5Y cells. Translatability of exon IIa 5′ UTR, however, was different in these two systems, suggesting cell type–specific effects governing its translational regulation. In addition to marked repression in luciferase assays, exon IIc–containing *Bdnf* mRNA also showed lower association with ribosomes in TRAP analysis, suggesting lower translatability. The physiological relevance of exon II–containing *Bdnf* transcripts has been highlighted in previous studies. First, *Bdnf* exon II–associated promoter is regulated by huntingtin (69), a protein causally linked to Huntington's disease. In addition, it was recently demonstrated that *Bdnf* promoter II KO mice (also promoter I, but not promoter IV and VI knockouts) displayed a strong hyperphagic obesity phenotype (70). In a series of studies, it has been shown that exon II-*Bdnf* mRNAs are targeted to proximal dendrites in rat hippocampal neurons (22–24). Our results support the possibility that translation of these transcripts is poised for translational activation by a currently unknown stimulus.

Alternative 3′ UTRs allow an additional layer of post-translational regulation, acting as a scaffold for binding miRNAs and RNA-binding proteins (RBPs). Through combinations of RNA–RNA and RNA–protein interactions, 3′ UTRs can regulate different processes such as mRNA stability, subcellular localization, and efficiency of translation (59). Recent studies have shown further potential functions for 3′ UTRs such as protein localization (71) or generation of non-coding RNA fragments (72). It is believed that long 3′ UTRs predominantly act as translational repressors by recruiting miRNAs and RBPs (73, 74). The results of our study suggest a more complicated regulation led by *Bdnf* 3′ UTRs and reveal a

nonrepressive role in BDNF expression in the brain. First, luciferase assays and TRAP experiments showed that the long *Bdnf* 3′ UTR does not have a repressive role on translation in cultured neurons. Second, and in line with this, 3′ UTR–long isoform promoted higher levels of *de novo* reporter protein synthesis in HEK293 cells as detected by pulldown of *de novo*–synthesized proteins. Third, *in vivo* experiments with conditional *Bdnf* 3′ UTR replacement with the 3′ UTR from *bGH* (bGH 3'UTR with a polyadenylation site, bGHpA) revealed lower BDNF levels in the brain. 3′ UTR replacement with bGHpA has been used as a strategy for upregulating protein expression *in vitro* and *in vivo* (38). Comparison of 27 3′ UTRs in mouse embryonic stem (ES) cells using knock-in alleles highlighted bGHpA—the same version as used in our study—as the strongest expression-promoting 3′ UTR (38) and in line with this, substitution of *Tgfβ1* or *Gdnf* 3′ UTRs by the *bGH* 3′ UTR resulted in hypermorphic mice (75, 76). Surprisingly, *Bdnf* 3′ UTR replacement with bGHpA resulted in lower BDNF protein expression in all tested brain regions (including cortex, striatum, thalamus, and hypothalamus) but elicited a hypermorphic effect in the lung and heart, suggesting that different 3′ UTR–targeted regulation mechanisms are at play in regulating BDNF expression in neuronal and non-neuronal tissues. In hippocampal neurons, different RBPs have been shown to mediate activity-dependent transport of *Bdnf* 3′ UTR–short and 3′ UTR–long mRNAs to dendrites (77). It is possible that a different set of RBPs or miRNAs bind and regulate the expression the BDNF 3′ UTR in neuronal and non-neuronal tissues.

Regulation of protein synthesis by BDNF in neurons is well described (78, 79). In contrast, it is not well known how synthesis of BDNF protein itself is regulated by neuronal activity and other stimuli. UTRs have been shown to affect the efficiency of protein synthesis by a variety of mechanisms (59). Vaghi *et al*. (25) investigated the effects of *Bdnf* 5′ UTRs, 3′ UTRs, or different combinations of these on reporter translation in neuroblastoma SH-SY5Y cells, studying reporter translatability as a response to a panel of excitatory neurotransmitters (47). The authors found that neither 5′ nor 3′ UTRs alone had a large effect on stimulus-dependent reporter translation, but combinations of 3′ UTR long and 5′ UTR IIc or VI showed facilitated translation as a response to stimuli (25). In another study, Lau *et al*. showed that the long *Bdnf* 3′ UTR repressed reporter translation at rest in hippocampal neurons but facilitated translation upon treating neurons with an long-term potentiation-inducing agent, tetraethylammonium (31). These authors also showed that a short (30 min) pulse of pilocarpine-induced neuronal activation induced a shift of 3′ UTR long–containing *Bdnf* mRNA from monosomal to polyribosomal fractions in the hippocampus (31). In the current study, we investigated neuronal activity–dependent *Bdnf* translation in cultured hippocampal and cortical neurons using UTR reporters and KCl depolarization. The treatment regimen (55 mM KCl for 15 min or 3h) was based on a previous report showing increased BDNF protein content in hippocampal neurons (47). The latter study also showed the upregulation of GFP signal from *Bdnf* VI-5′ UTR–GFP-3′ UTR–long reporter in the dendrites of hippocampal neurons upon 3h KCl stimulation, suggesting the exon VI-3′ long combination as a variant in the *Bdnf* UTR "code" enabling stimulus-dependent translation (47). Here, we measured modest activity-dependent increase in luciferase signal after 3h KCl treatment for 5′ UTR II(c) reporter construct in cortical neurons and for 5′ UTR VI, 3′ UTR short, and 5′ UTR VI/3′ UTR–long combinations in hippocampal neurons. However, in our hands, a robust stimulus-dependent translation manifesting in both cultures was not detected with any single-UTR construct (5′ or 3′ UTR variant alone) nor with a construct using a 5′ UTR VI/3′ UTR–long combination. Future studies are needed to determine the exact signaling cues regulating translation of mRNAs containing these UTRs.

In parallel with studying translational control over *Bdnf* UTR reporters, we wanted to know if we could detect activity-dependent translation of the BDNF protein in cortical neurons. We used a short range of time points (5–15 min) to detect a possible increase in BDNF protein by translational control, since longer time points would have introduced a confounding effect from activity-dependent *Bdnf* transcription (16). Two studies have reported very rapid stimulus-dependent upregulation of BDNF protein in cultured neurons by KCl treatment (47) or in the hippocampus by intraperitoneal ketamine injection (51). Here, we readily detected increase in BDNF protein, three hours after neuronal activity stimulation (likely reflecting neuronal activity–dependent transcription). However, no increase in BDNF protein was detected at 5 to 15 min of stimulation, showing that neuronal activity does not induce robust BDNF protein translation. It has to be noted that measuring activity-dependent BDNF regulation in these experiments is not straightforward as BDNF secretion is also regulated by neuronal activity (26, 80), and KCl or glutamate treatments may cause its release from the intracellular pool. To circumvent this, *Bdnf* translation could be addressed with methods for detecting *de novo*–synthesized protein such as OPP-ID (44); we have attempted this but have not reached the necessary sensitivity for detecting endogenous newly synthesized BDNF in cultured neurons.

SNs isolated from intact brain have been shown to contain a rich transcriptome, as revealed by a recent study combining synaptosome fractionation with fluorescence-activated sorting (81). Both *Bdnf* mRNA (82) and BDNF protein (83) have been detected in SNs, but to the best of our knowledge, comparative analysis of alternative BDNF transcripts in SNs has not been performed. We found that all major *Bdnf* alternative transcripts were highly enriched in rat hippocampal and cortical SNs, suggesting that the targeting mechanism may rely on the commonly used sequence in exon IX. Although the differences in enrichment between alternative *Bdnf* transcripts were modest, we observed higher enrichment for exon I–containing transcripts. Leaving aside the debate about dendritic localization of *Bdnf* mRNA as such (for example, see (29, 30) vs (22–24)), exon I-transcripts have previously been associated with somatic localization (22–24). BDNF protein has been detected in presynaptic vesicles (30), suggesting that the observed enrichment in SNs may reflect axonal targeting of

Bdnf mRNA. Previously, we have shown enhanced translation of BDNF protein from an alternative start codon in exon I (26), which could be used to potentiate BDNF function in subcellular loci accumulating higher amounts of exon I-*Bdnf* mRNA. The contribution of exon I-*Bdnf* mRNA to the synaptic functions of BDNF protein needs to be tested in future studies.

Taken together, our analysis of *Bdnf* UTRs contributes new insights to understanding post-transcriptional regulation of *Bdnf* expression. We found that alternative 5′ UTRs have a variable but generally repressive effect on reporter translatability. Second, we discovered that *in vivo*, the *Bdnf* 3′ UTRs have a facilitating role on BDNF expression in the brain but show a repressive effect in peripheral tissues. Third, no evidence was obtained for robust activity-dependent translation of BDNF mRNAs with different UTRs. Finally, the SN results add to the understanding of subcellular localization of alternative *Bdnf* mRNAs.

Experimental procedures

Cell culture

Rat primary neuron cultures were prepared from E21 rat embryos (Sprague-Dawley) as described previously (84), with relevant procedures approved by the ethics committee of animal experiments at the Ministry of Agriculture of Estonia (Permit Number: 45). Briefly, cortical or hippocampal tissue was dissected and preserved in Leibovitz L15 media (PAA Laboratories) until further processing. Tissue was dissociated in 1 ml of 0.25% Trypsin + 1 mM EDTA (Invitrogen) at 37 °C for 20 min, with 0.5 mg/ml DNase I and 12 mM $MgSO_4$ added for the last 10 min. 275 μl of 1% trypsin inhibitor (Invitrogen), 110 μl of 10% bovine serum albumin (Pan-Biotech), and 50 μl of DNase I (stock solution 5 mg/ml, Roche Diagnostics) were added and tissue was triturated with a 1 ml pipette tip. Tissue suspension was diluted 5× in 1 × Hank's Balanced Salt Solution and centrifuged at 200*g* for 30 s to remove tissue chunks and cells collected by centrifugation at 200*g* for 6 min. Cells were grown in Neurobasal A (PAA Laboratories or Invitrogen), supplemented with 1 × B27 Supplement (Invitrogen), 100 U/ml Penicillin + 0.1 mg/ml Streptomycin (Invitrogen), or 100 μg/ml Primocin (InvivoGen) and 1 mM L-glutamine (Invitrogen). At 2 DIV, half of the growth medium was changed and mitotic inhibitor 5-fluoro-2′-deoxyuridine (Sigma Millipore) was added to prevent glial proliferation. HEK293 cells were grown in DMEM medium (PAA Laboratories) supplemented with 10% Fetal Bovine Serum (PAN Biotech), 100 U/ml Penicillin + 0.1 mg/ml Streptomycin (Invitrogen).

DNA constructs

Bdnf UTR sequences were amplified from genomic DNA of cultured rat hippocampal neurons using Phusion Hot Start High-Fidelity DNA polymerase (35 cycles) and primers listed in Table S1. Sense primers were designed to match the alternative transcription start sites in 5′ UTRs or the beginning of the 3′ UTR. Antisense primer for 5′ UTR sequences matches the noncoding 5′ sequence in the coding exon IX shared by all *Bdnf* transcripts, except for exon I transcripts where antisense primer targeted the sequence preceding the exon I–encoded ATG. All UTR sequences were subcloned into the pTZ57R/T plasmid (Thermo Fisher Scientific) and verified by sequencing. Next, UTR sequences were cloned between HindIII/NcoI (for 5′UTR constructs) or XbaI/SalI (for 3′UTR constructs) in a pGL4.15[luc2P/Hygro] (Promega) vector, where the promoter had been replaced with the elongation factor 1 alpha (EF1a) promoter sequence. Therefore, resulting constructs contained (under the control EF1a promoter) either *Bdnf* 5′ UTR preceding or *Bdnf* 3′ UTR following the reporter sequence. Alternative 5′ UTRs were cloned between the EF1a promoter and firefly luciferase, with an SV40 poly(A) cassette 3′ of the luciferase ORF, generating a 0.17 kb 3′ UTR. For testing *Bdnf* 3′ UTRs, *SV40* poly(A) was replaced with short (0.8 kb) or long (3 kb) *Bdnf* UTR (Figs. 1*A* and S1*A*). As a control, we used a construct with the *SV40* poly(A) and without a *Bdnf*-derived 5′ UTR. The *Renilla* luciferase construct contained the same 5′ and 3′ UTR sequences as the firefly control plasmid. VI-2 construct was cloned by inserting a SacI/NcoI fragment from VI-1 construct into HindIII/NcoI sites of pGL4.15[luc2P/Hygro]EF1a vector using blunt/NcoI ligation. VI-1 ATG→GTG mutant construct was obtained by primer-directed mutagenesis of exon VI-1 plasmid (using primers shown in Table S1 and Phusion polymerase).

Transfections and luciferase assays

Rat primary cortical neurons were transfected with *Bdnf* luciferase constructs using Lipofectamine 2000 (Thermo Fisher Scientific) following the manufacturer's instructions. A DNA/Lipofectamine ratio of 1:2 (w:v) was used for transfections. *Renilla* luciferase reporter under EF1a promoter control was cotransfected with *Bdnf* UTR/firefly luciferase constructs in 1:1 ratio for normalization. Hundred nanograms of total plasmid per well in a 96-well plate (luciferase assays) and 800 ng of plasmid per well in a 12-well plate (RNA analysis) were used. After 3 h, the transfection medium was replaced with the medium removed from the same cultures before transfection. 48 h after transfection, cells were lysed in Passive Lysis Buffer (Promega) and used for Dual-Glo luciferase reporter analysis according to the manufacturer's instructions. Luciferase signal was measured using the GENios Pro plate reader (Tecan). Average values of duplicate transfections were used for luciferase assay for each biological replicate; single transfections were used for each biological replicate for RNA analysis. Transfections for luciferase assays and reporter RNA analysis were processed simultaneously.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from cortical neurons using RNeasy Micro Kit (Qiagen) according to manufacturer's instructions. To efficiently eliminate genomic DNA from RNA, DNase-digestion was carried out after RNA purification in solution, rather than during the RNA extraction procedure on spin-columns. To this end, Turbo DNA-free Kit (Ambion) was used, following the manufacturer's instructions. For reverse transcription, 1 μg of total RNA, oligo(dT) primer, and

Superscript III First-Strand synthesis system (Invitrogen) were used according to manufacturer's instructions. Quantitative real-time PCR was performed using LightCycler 480 II (Roche), LightCycler 480 SYBR Green I Master reaction mix (Roche), and primers shown in Table S1. Firefly luciferase mRNA expression levels were normalized to the levels of Renilla luciferase.

Translating ribosome affinity purification

Rat E21 hippocampal neurons were seeded in 6-well plates at the density of 50.000 cells/cm^2 and transduced with AAV-[Syn]-Rpl10a-eGFP or AAV-[Syn]-eGFP control virus ($\sim$10^9 vg/ml) at 6 days *in vitro*. Preparation of AAVs was performed as described previously (26). AAV plasmids encoding Rpl10a-eGFP and eGFP under the neuron-specific human Synapsin I [Syn] promoter were kindly provided by Priit Pruunsild and Hilmar Bading (University of Heidelberg). Cells from three wells infected with either virus were combined for one pull-down. At 2 weeks in culture, cells were washed twice with 1 ml ice-cold PBS containing 100 μg/ml cycloheximide and lysed in 500 μl lysis buffer containing the following in RNAse-free water: 10 mM Hepes–KOH (pH 7.4), 5 mM MgCl2, 150 mM KCl, 1% NP-40, 0.5 mM DTT, 1× protease inhibitor cocktail (Roche Mini Complete, EDTA-Free), 1 mM PMSF, and 400 U/ml RiboLock RNase Inhibitor (Thermo Fisher Scientific). Lysates were collected by scraping and kept on ice for 30 min. Cell debris was pelleted by centrifugation in a tabletop centrifuge at 4 °C for 10 min at maximum speed. Two-third of the cleared lysates was used for immunoprecipitation (IP) and one-third for preparing input samples. 75 μl of Protein G Dynabeads (Thermo Fisher Scientific) were used per IP. Beads were washed twice in 500 μl of lysis buffer and incubated in 500 μl of lysis buffer with 5 μg of anti-GFP antibodies (2.5 μg of clone Htz-GFP-9C8 and 2.5 μg of clone Htz-GFP-19F7, Memorial Sloan-Kettering Monoclonal Antibody Facility) for 1 h at 4 °C with end-over-end mixing. Antibody-bead complexes were washed with 500 μl of lysis buffer, resuspended in 100 μl of lysis buffer, and added to the lysates. Bead-antibody complexes were incubated with lysates for 1 h at 4 °C on a rotator mixer, followed by 3× beads washes with 500 μl of wash buffer. RNA was extracted from the beads using RLT buffer from the Qiagen RNeasy Micro kit and purified according to the manufacturer's instructions. Complementary DNA was synthesized from 200 ng of input RNA and 10 μl of IP RNA (equal volumes were used for pulldowns from Rpl10a-eGFP and control eGFP virus) using oligo(dT) primers and Superscript III. The efficiency of ribosome-associated RNA pulldown efficiency was estimated by the enrichment ratio of Rpl10a-eGFP^{IP}/eGFP^{IP} in qPCR, found to be in the 10^2-10^3 range for all amplicons.

OPP labeling, biotinylation, and streptavidin pulldown of newly synthesized proteins

HEK293 cells were transfected with *Bdnf* luciferase constructs using PEI in a 2:1 DNA/PEI ratio. Ten micrograms of plasmid were used per 10 cm dish. Twenty four hours after transfection, cells were labeled with 20 μM OPP for 1 h, washed twice with ice-cold PBS, and lysed in click lysis buffer (100 mM Hepes pH 7.5, 150 mM NaCl, 1% NP-40, 2 mM PMSF, and EDTA-free protease inhibitors (Thermo Fisher Scientific, A32965)). OPP-labeled proteins were then tagged with biotin by click chemistry, following Terenzio *et al*. 2018 (85), with minor modifications. Reactions were carried out at RT for 2 h, under gentle rotation, in the presence of 1% SDS, 0.1 mM Dde Biotin Picolyl Azide (Click Chemistry Tools, 1186-5), 0.1 mM Tris(3-hydroxypropyltriazolylmethyl)amine, 1 mM Tris(2-carboxyethyl)phosphine hydrochloride, and 1 mM $CuSO_4$. In control reactions, biotin azide was omitted. Click-conjugated proteins were precipitated with five volumes of acetone, overnight at −20 °C, pellets washed with ice-cold methanol and resuspended in PBS-1% SDS-1× protease inhibitors. Protein concentration was determined using the Pierce bicinchoninic acid assay Protein Assay Kit (Thermo Scientific). For streptavidin pulldowns, equal amounts of protein (200–300 μg) were incubated with 15 μl of Pierce Streptavidin Magnetic Beads (Thermo Fisher Scientific, 88817) in PBS-0.2% SDS-1% NP-40 for 2 h, at RT with gentle rotation. Beads were then washed twice with PBS-0.2% SDS-1% NP-40 for 10 min at 4 °C, three times with PBS-6M urea-0.1% NP-40 for 30 min at 4 °C, once with PBS-0.2% SDS-1% NP-40 for 10 min at 4 °C, and once with PBS at RT for 1 to 2 min before elution with 1× Laemmli buffer for 5 min at 95 °C. Equal amounts of protein or equal volumes from eluted fractions were analyzed by SDS-PAGE.

Protein extraction and Western blot

Dissected tissue samples were weighed and 7 μl of ice-cold RIPA (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 1× cOmplete protease inhibitor cocktail (Roche)) per 1 mg of tissue was added. The tissue was homogenized by trituration with pipette tip and then SDS was added to a final concentration of 0.5%. For efficient protein extraction, the lysate was sonicated using BioRuptor Pico device (Diagenode) and rotated at 4 °C for 20 min. Finally, the insoluble material was removed by centrifugation at 16,000*g* at 4 °C for 20 min. Protein concentrations were measured with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific), and 50 μg of total protein were loaded to SDS-PAGE for BDNF Western blot analysis along with recombinant mature BDNF protein (Icosagen, cat. no P-105-100) as a positive control. For heart and lung tissue, 10 μl of ice-cold RIPA per 1 mg of tissue was used and tissue was homogenized using the Potter-Elvehjem homogenizer. BDNF IP was performed to measure BDNF levels. For that, 1 mg of protein lysate in RIPA was diluted to 1 ml, achieving 1 mg/ml protein concentration and achieving 0.1% SDS final concentration. Two micrograms of BDNF antibody (mAb#9, a kind gift from Y. A. Barde, Cardiff University) was added to each IP sample and incubated overnight at 4 °C. The next day, the antibodies were captured using 25 μl of Dynabeads Protein G (Invitrogen) per IP for 3,5 h at 4 °C. An aliquot of unbound proteins (flow-through samples) were collected as a control of equal protein levels. Next, the

beads were washed 4 times with RIPA supplemented with 1× cOmplete protease inhibitor cocktail (Roche) and 0.1% SDS. The immunoprecipitated proteins were eluted with 1× Laemmli buffer with 5% β-mercaptoethanol at 95 °C for 5 min. Equal amounts of IP material and flow through samples were analyzed using SDS-PAGE. SNs and cultured cells were lysed directly in Laemmli buffer (10% β-mercaptoethanol, 2% SDS, 10% glycerol, 0.0625 M Tris–HCl pH 6.8).

Proteins separated by SDS-PAGE were transferred to PVDF membranes using Trans-Blot Turbo Transfer system (Bio-Rad). The following antibodies were used for immunoblot: anti-firefly luciferase (Novus Biologicals, NB100-1677, 1:1000), anti-BDNF (Icosagen, clone 3C11, 1:1000), anti-PSD-95 (Thermo Fisher Scientific, MA1-046, 1:2000), anti-CaMKIIα (Millipore, 05-532, 1:2000), anti-GAPDH (Millipore, MAB374, 1:5000), anti-AcH3 (Millipore, 06-599, 1:1000), anti-Synapsin 1 (Synaptic Systems, 106103, 1:5000), HRP-conjugated secondary antibodies (anti-mouse IgG, Thermo Fisher Scientific; anti-goat IgG, Santa Cruz Biotechnology) were used at a 1:5000 dilution. Immunoblots were developed with Super-Signal West Pico/Atto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and images captured with Image-Quant LAS 4000 imaging system (GE Healthcare Life Sciences), ensuring that all imaged bands were unsaturated and in a linear range of detection. For equal loading control, membranes were stained with Coomassie Brilliant Blue R-250 Dye. Signal intensity quantification was performed using the ImageQuant TL software (ImageQuant TL 8.2, https://cdn.cytivalifesciences.com/api/public/content/digi-45099-pdf).

Generation and analysis of Bdnf *3′ UTR replacement knock-in mice*

The targeting construct for the *Bdnf* conditional 3′UTR replacement allele (c3′ R) was generated by Cyagen. The targeted allele contains a 1382 bp 5′ homologous arm followed by a neomycin resistance gene flanked by rox sites, a 610 bp cassette containing the bGHpA signal in an inverted orientation flanked by the FLEx system (45) starting immediately after the stop codon, and a 5155 bp 3′ homologous arm. G4 ES cells were electroporated with 30 μg of linearized targeting construct followed by neomycin selection, PCR screening, and Southern blot confirmation. Correctly targeted ES cells were microinjected into C57BL/N6Crl mouse blastocysts to generate chimeric mice. Germline transmission was achieved by breeding male chimeras with C57BL/N6Crl females. The bGHpA sequence was removed using the CAG-Flp mouse line at the F2 generation. Mice carrying the *Bdnf* c3′ R allele were crossed with a ubiquitous Pgk1-Cre mouse line, where Cre expression results in ubiquitous recombination (46). Mutant pups were identified as positive by PCR screening using primer pairs for three different regions of the *Bdnf* c3′ R allele, including the genotyping primers shown in Fig. S2*A*. For experiments, genotyping samples were collected at weaning and during dissection. Genomic DNA was isolated from the tail using Extracta DNA Prep for PCR - tissue (Quanta Biosciences). Genotyping was performed using AccuStart II GelTrack PCR SuperMix (Quanta Biosciences) and analyzed by electrophoresis using 1.5 to 2% agarose gels in Tris-acetate-EDTA buffer. The primers used for genotyping the mice for the presence of *Bdnf* c3′ R allele are shown in Table S1. Mice were maintained at temperature-controlled conditions of 20 °C to 22 °C under a 12 h/12 h light/dark cycle at a relative humidity of 50 to 60%. Cages and bedding material (aspen chips, Tapvei Ou) were changed every week, and wooden tube and aspen shavings were provided as enrichment. Mice received food and water *ad libitum*. Animal experiments were conducted following the 3R principles of the EU directive 2010/63/EU, which govern the care and use of experimental animals, and were approved by the County Administrative Board of Southern Finland (license numbers ESAVI-2010-09011/Ym-23 and ESAVI/11198/04.10.07/2014). The protocols were authorized by the national Animal Experiment Board of Finland. Mice were euthanized at 2-3 months of age by cervical dislocation followed by decapitation after deep anesthesia with CO_2. Both males and females were used for experiments. The brain areas of interest were dissected using a scalpel or a puncher (2 mm inner diameter). Brain tissues isolated from one specific hemisphere (left or right) were used to perform mRNA and protein analyses and were always matched for all the animals in all the experiments performed. Peripheral tissues were collected using a scalpel. After collection, tissues were snap frozen and stored at −80 °C until processed.

SN preparation

SNs were prepared from the cerebral cortices and hippocampi of 2 to 3–month old adult male Sprague Dawley rats as previously described in De Rubeis and Bagni (63). Brain tissue was dissected and prepared on ice, with solutions and tools precooled to +4 °C. Dissected cortices or hippocampi were homogenized at 4 °C with a Dounce Homogenizer (Wheaton) in homogenization buffer (5 mM Hepes pH 7.4, 1 mg/ml BSA, 1 mM EDTA, 0.32 M sucrose). After homogenization with eight strokes, total unfractionated lysate fraction (T) was collected. Homogenized tissue was centrifuged at 3000*g* for 10 min for the precipitation of nuclei and unlysed material, and the supernatant was collected, comprising the cytosolic and SN fraction (S1). The S1 fraction was centrifuged at 14,000*g* for 12 min, and the cytosolic fraction was collected from the supernatant (S2). The precipitate was resuspended in Hepes–Krebs buffer (147 mM NaCl, 3 mM KCl, 10 mM glucose, 2 mM $CaCl_2$, 20 mM Hepes (pH 7.4), 2 mM $MgSO_4$); supplemented with 120 U/ml RiboLock RNAse inhibitor (Thermo Fisher Scientific) when preparing fractions for RNA extraction. 0.8 volumes of Percoll (Sigma Aldrich) were added to this fraction and centrifuged at 14,000*g* for 2 min. SN fraction was collected from supernatant, resuspended in Hepes–Krebs buffer, and centrifuged at 14,000*g* for 30 s to remove excess Percoll. Precipitated SN fraction was recovered and resuspended in Hepes–Krebs buffer.

Statistical analysis

All data are expressed as means ± SD. Two-tailed Student's *t* test was performed when comparing two groups, one sample *t* test when comparing a population mean with a reference value

and one-way ANOVA when comparing three or more groups. Statistical analysis was performed using GraphPad Prism or Microsoft Excel software. p values < 0.05 were considered statistically significant.

Data availability

All relevant data are contained within the article.

Supporting information—This article contains supporting information (14).

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: AAV, adeno-associated virus; BDNF, brain-derived neurotrophic factor; bGH, bovine growth hormone; bGHpA, bGH polyadenylation; ES, embryonic stem; IP, immunoprecipitation; OPP, O-propargyl puromycin; RBP, RNA-binding proteins; TRAP, translating ribosome affinity purification.

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