Pharmacological Profile of Brain-derived Neurotrophic Factor (BDNF) Splice Variant Translation Using a Novel Drug Screening Assay
A “QUANTITATIVE CODE”*

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The neurotrophin brain-derived neurotrophic factor (BDNF) is a key regulator of neuronal development and plasticity. BDNF is a major pharmaceutical target in neurodevelopmental and psychiatric disorders. However, pharmacological modulation of this neurotrophin is challenging because BDNF is generated by multiple, alternatively spliced transcripts with different 5′- and 3′-UTRs. Each BDNF mRNA variant is transcribed independently, but translation regulation is unknown. To evaluate the translatability of BDNF transcripts, we developed an in vitro luciferase assay in human neuroblastoma cells. In unstimulated cells, each BDNF 5′- and 3′-UTR determined a different basal translation level of the luciferase reporter gene. However, constructs with either a 5′-UTR or a 3′-UTR alone showed poor translation modulation by BDNF, KCl, dihydroxyphenylglycine, AMPA, NMDA, dopamine, acetylcholine, norepinephrine, or serotonin. Constructs consisting of the luciferase reporter gene flanked by the 5′-UTR of one of the most abundant BDNF transcripts in the brain (exons 1, 2c, 4, and 6) and the long 3′-UTR responded selectively to stimulation with the different receptor agonists, and only transcripts 2c and 6 were increased by the antidepressants desipramine and mirtazapine.

We propose that BDNF mRNA variants represent “a quantitative code” to regulate expression of the protein. Thus, to discriminate the efficacy of drugs in stimulating BDNF synthesis, it is appropriate to use variant-specific in vitro screening tests.

The neurotrophin brain-derived neurotrophic factor (BDNF),4 a key morphoregulatory molecule in neuronal development and plasticity (1, 2), has recently been identified as one of the four major pharmaceutical targets in neuropsychiatric diseases, including depression and bipolar disorder (3–5). In addition, BDNF is increasingly considered as a potential therapeutic agent for neurodevelopmental disorders such as Rett syndrome (6). However, the complex regulation of the bdnf gene complicates any attempt to modulate this neurotrophic factor through a pharmacological intervention. Indeed, transcription of the bdnf gene produces 11 primary transcripts in rodents (7) and 17 in humans (8), and each is characterized by a different 5′-untranslated (UTR) exon linked by alternative splicing to a common exon encoding the protein and the 3′-UTR. Because the 3′-UTR contains two polyadenylation sites, each primary transcript can exist in two forms, one with a short and the other with a long 3′-UTR, producing a total of 22 (in rodents) or 34 (in humans) possible transcripts (7, 8). In this context, new approaches taking into account the intrinsic biological complexity of BDNF are required to develop a pharmacological strategy to regulate BDNF levels.

The different BDNF splice variants are transcribed independently from each other (9), and several antidepressants and mood stabilizers were shown to have a modulatory effect on transcription of BDNF variants, with a different profile depending on the drug used (10–13). However, because a complete description of rodent bdnf and human BDNF gene structure and splice variants was not available until 2007, all studies on BDNF transcripts expression related to a specific disease or treatment carried out before this date are incomplete.

Pruunsild et al. (8) and Aid et al. (7) showed that the 5′-UTR region of each BDNF transcript is encoded by a different exon with unique length, GC content, and putative secondary structures. Accordingly, we hypothesized that each BDNF transcript may display a different translatability. However, because the final protein product is the same, it is presently impossible to determine in vivo in wild-type animals the relative contribution

*The abbreviations used are: BDNF, brain-derived neurotrophic factor; Fluc, firefly luciferase; Rlu, Renilla luciferase; DHPG, dihydroxyphenylglycine; ACh, acetylcholine; NE, norepinephrine; 5-HT, serotonin; RLU, relative light unit; uORF, upstream open reading frame; nt, nucleotide; CDS, coding sequence.
of single BDNF splice variants to the production of the BDNF protein. In YAC or BAC transgenic mice in which the BDNF coding region was replaced with GFP or the lacZ reporter gene to visualize variant-driven expression of BDNF, the reporter protein could not be detected in the brain (14–16). In addition, the role of the different 5'UTRs and 3'UTRs in regulating BDNF translation is presently unknown. Thus, to determine the role of BDNF transcripts in producing the protein, it is necessary to develop new tools able to determine the amount of protein generated by each BDNF splice variant (with different 5'UTRs and 3'UTRs) at basal conditions and in response to a disease state or a specific drug. Considering that a change in the mRNA levels of one transcript that is poorly translated will have less impact on total BDNF levels than a highly translatable splice variant, the current issue in BDNF drug discovery is to determine which compounds are able to modulate BDNF synthesis from the different transcripts. Here, using a novel in vitro assay, we characterized the pharmacological profile for translation of all currently known rat bdnf transcripts in response to the major excitatory neurotransmitters.

**EXPERIMENTAL PROCEDURES**

**Cloning and Generation of Luciferase Constructs pN1-RLuc and pN1-FLuc**—pN1-RLuc and pN1-Fluc were obtained by the replacement of the enhanced GFP coding sequence of pEGFP-N1 (Clontech), respectively, with the Renilla luciferase (GI:2582516; GB:AA882577.1 from the vector pRL-SV40) or with the Firefly luciferase open reading frame (ORF) (GI: 13195704; GB:AAB89084.1, from the vector pGL3 basic). The cloning strategy and the resulting maps of the newly obtained pN1-RLuc (pRLuc) and pN1-Fluc (or pFluc) are shown in Fig. 1. pN1-RLuc (or pRLuc) was used as transfection control and for normalization, whereas pN1-Fluc (pFluc) was also used as a backbone to construct the vectors containing the different BDNF splicing variants. The Rluc and Fluc ORFs were amplified with Phusion high fidelity DNA polymerase (Finnzymes) using PCR conditions and primer sequences that are shown in Table 1. The sequence of single rat BDNF exons 1–8 encoding the alternatively spliced 5'UTR regions (Fig. 1a) was amplified by PCR starting from the adult rat brain cDNA. cDNA from RNA-extracted and retro-transcribed using TRIzol (Invitrogen) and Superscript-II transcriptase (Promega) was prepared following the manufacturer’s instructions. The forward primers specific for each 5' exon, are reported in Table 1 (Eurofins MWG GmbH). PCR amplicons were cloned in the pRLuc-N1 vector upstream of the Firefly luciferase coding sequence. Separately, the complete rat bdnf-coding sequence (CDS) was cloned in the pFluc-N1 vector upstream from the Fluc gene. Furthermore, the two 3'UTR region variants, called 3'UTRlong and 3'UTRshort, respectively, were cloned into the pN-Fluc vector downstream from the Fluc gene generating pN1-Fluc-3'UTRlong/short). Finally, the two Kozak sequences, one specific for exon 1 and one common to all other transcripts (exon 9), were inserted through a modified Fluc sequence. To obtain bdnf exons 1, 2c, 4, or 6, joined with Fluc and BDNF 3'UTRlong, the pN1-Fluc-3'UTRlong was used as backbone, and bdnf exons were cloned generating the pN1-insert name-Fluc-3'UTRlong vectors.

**Cell Culture and Transfection**—Human neuroblastoma cell lines SH-SY5Y were grown in Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 g/ml streptomycin at 37 °C with 5% CO₂. The different pN1-Fluc constructs were transfected into SH-SY5Y cells using Lipofectamine 2000™ (Invitrogen) according to the manufacturer’s protocol. One day prior to transfection, SH-SY5Y cells were seeded into a 96-well white plate with a clear bottom at a confluence of 50 – 60% and cultured overnight in DMEM, 10% FBS. The relative quantities of DNA and transfectant agent were determined empirically, and the optimal ratio was found to be at 0.2 μg of DNA and 0.5 μl of Lipofectamine for each well. Each Fluc-containing construct was transfected together with the normalized Rluc construct in a 10:1 ratio.

**Dual-Luciferase Assay**—Luciferase assay was performed according to the manufacturer’s instructions (Promega). In brief, 24 h after construct transfection, the growth medium was removed, and cells were washed once with phosphate buffer at pH 7.2. The cell lysis was performed adding 20 μl of passive lysis buffer into each well and placing the plate in gentle agitation at room temperature for 15 min. After uniform lysis, 100 μl of Luciferase Assay Reagent II (LARII) were dispensed in each well, and Firefly luciferase activity was measured using the GloMax-Multi luminometer with two injectors (Promega Corp.). After quantifying the Firefly luminescence, this reaction was quenched, and simultaneously, the Renilla luciferase reaction was initiated by adding 100 μl Stop & Glo Reagent (Promega) to the same well, and then Renilla luciferase activity was measured. The ratiometric value of Firefly luciferase on Renilla luciferase in the experiments with 5'UTR regions of BDNF in fusion with the Firefly luciferase sequence were normalized to the Firefly mRNA levels measured through semiquantitative PCR.

**Cells Treatments**—The Dual-Luciferase assay was used to evaluate the effects of different receptor agonists and antidepressant drugs on the bdnf variant translation in SH-SY5Y cells. A comprehensive characterization of neuronal receptor expression by SH-SY5Y cells was recently shown by Korecka et al. (17). The cells were treated for 3 h with the listed compounds at the following concentrations: KCl (50 mm, Sigma) (18); BDNF (50 ng/ml, Sigma) (19); (S)-3,5-dihydroxyphenylglycine (DHPG, 50 μM, Ascent Scientific) (20); AMPA (30 μM, Ascent Scientific) (21); NMDA (30 μM, Ascent Scientific) (22); acetylcholine (ACh, 30 μM, Sigma) (23); norepinephrine (NE, 50 μM, Sigma) (24); dopamine (40 μM, Sigma) (25); serotonin (5-HT, 50 μM, Sigma) (26) or desipramine 10 μM (Abcam) (27); and mirtazapine 50 μM (Abcam) (28). The usage of these agonists is reported in the literature with a very broad range of concentration and timing. Therefore, we selected the different amounts upon a proven biological activity on SH-SY5Y and the lack of possible toxic effects (see Fig. 3a). To have a sure and reliable drug effect on protein translation, we treated the cells for 3 h. This time length was chosen with the goal of maximizing the drug effect on protein translation, we treated the cells for 3 h. This is of single addition of agonists to experimental operations (i.e. the single addition of agonists in cell cultures). All treatments were performed in a bath for 3 h, in accordance with previous evidence showing that this time frame is sufficient to have a robust activity-dependent production of BDNF proteins in vivo (29, 30).
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Viability Assay and Western Blot—The different drugs were tested in a viability assay on SH-SY5Y cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) following the manufacturer’s instruction. Western blot analysis was used to detect the Firefly luciferase amount in SH-SY5Y cells.

| Primer | Primer Forward | Primer Reverse | PCR CONDITIONS |
|--------|----------------|----------------|----------------|
| Fluc   | GCCACCGTGTCGCCAC CATGGAAGACGCC | CGGGCGGGCGCTTACAC GGCAGCATTTCCGC | Denaturation 98°C 10 s Extension 72°C 40 s | 30 cycles |
| Rluc   | GCCACCGGTCCGCAC CATGACTCTCACAAGT TTATGATCC | CGGGCGGGCGCTGGGCG CGTTGTCTATTGATGTA ACTCG | Denaturation 98°C 10 s Extension 72°C 40 s | 30 cycles |
| 3’UTR long | GGGCGGGCCTGATTTATAG | GCCGGTAACCTACAATA GGCTTCGATG | Denaturation 98°C 10 s Extension 72°C 2 min | 31 cycles |
| 3’UTR short | GGGCGGGGCGCTGATTTATAG | GTCAGTACCCTATTATAT CATTACAAATATAG | Denaturation 98°C 10 s Extension 72°C 15 s | 31 cycles |
| CDS    | GCCGTCGAGATGACC ATCCCTTTTCTC | GTCAACAGGTTCGCCCTTA TAAATGTCAGTG | Denaturation 98°C 10 s Extension 72°C 15 s | 31 cycles |
| Exon 1 | AATTCTCTAGTAAAG CGGTAGCTGGGTG GTACG | GTCTACGAGTTCTGC CATCGACGTACGTTCG | Denaturation 98°C 10 s Extension 72°C 20 s | 31 cycles |
| Exon 2a| GATCTCTAGACTTTT GGCAAAGGCTCAGTCG GCAG | GCCACCGGTCTGGATG AGTACTACACCCCGAC | Denaturation 98°C 10 s Extension 72°C 40 s | 31 cycles |
| Exon 2b| GATCTCTAGACTTTT GGCAAAGGCTCAGTCG GCAG | GTTACGCTGGAGCTGGC AAGAATATCTACGCG | Denaturation 98°C 10 s Extension 72°C 15 s | 31 cycles |
| Exon 2c| GATCTCTAGACTTTT GGCAAAGGCTCAGTCG GCAG | GAACCCGTTTTGATG AGCCTACCCACCCGGT GCAG | Denaturation 98°C 10 s Extension 72°C 35 s | 31 cycles |
| Exon 3*| AGCTCTAGACTTTT GGCAAAGGCTCAGTCG GCAG | GTACTACGGCTCAGTCG ACTAGTAAAAATGATCAC AGAG | Denaturation 98°C 10 s Extension 72°C 20 s | 31 cycles |
| Exon 4 | AATTCTCTAGACACCA CTTCCCTACCTCAGG AG | GACCTACGGGTAGTCAGT ACTGTAAGGATGAAACAT CAAGGC | Denaturation 98°C 10 s Extension 72°C 20 s | 31 cycles |
| Exon 5 | GACTCTCGGAAAACC ATAACCCCGACACT CTGTTGATTCATT GCTACGG | GACCTCGGACTCCCGGC ACTCCCGACCCACAGA GCTAGAAGGCCAGCAGC | Denaturation 98°C 10 s Extension 72°C 15 s | 31 cycles |
| Exon 6** | GACTCTCGGACCATCGAAGCTCAACACGA GACC | GATCACCAGGCTCAGGGT CCACCAAAAGCTACGGG | Denaturation 98°C 10 s Extension 72°C 20 s | 31 cycles |
| Exon 7 | GTACTCTAGACTTTT GGCAAAGGCTCAGTCG GCAG | GTACCGGCTCAGACCTG TAAAGTGCAACATTTTCA ATCTCCTGCG | Denaturation 98°C 10 s Extension 72°C 15 s | 31 cycles |
| Exon 8 | GTACTCTAGACTTTT GGCAAAGGCTCAGTCG GCAG | GCATACGGGTACCACT TCTCCAGCTTTGGA CAGCTCC | Denaturation 98°C 10 s Extension 72°C 35 s | 31 cycles |

* Exon 3 was amplified using Platinum Taq DNA Polymerase High Fidelity (Invitrogen).
** Exon 6 has a high GC percentage, and the amplification was preceded by 8 min of denaturation at 98°C.
cells 24 h after transfection with pN1-exon-Fluc vectors. Proteins were subsequently extracted in cold (4 °C) lysis buffer containing 137 mM NaCl (Fluka), 20 mM Tris–HCl, pH 8.0, 0.75% Nonidet P-40, 10% glycerol, and a mixture of protease inhibitors, 1 mM PMSF, 10 μg/ml TELW, 4 μg/ml soybean trypsin inhibitor, 1 mM iodoacetamide, 1 mM spermidine. The cell suspension was further homogenized with a syringe, rocked for 30–45 min at 4 °C, and centrifuged at 10,000 × g for 8–10 min at 10 °C, and the Bradford assay was used to determine protein concentration. Immunoblot was performed with anti-luciferase 1:1500 (Sigma) and mouse anti-α-tubulin 1:20,000 (Sigma) antibodies.

Statistical Analysis—We calculated that even with a S.D. of 20%, we were able to detect a 25% significant variation already after three repetitions with a statistical power of 70% (α error level of 5%). The final experiments using the full constructs were performed in quadruplicate to reach the statistical power of 80% required for future assay validation. These conservative parameters allowed us to claim as significant only the modulation in translatability characterized both by significant variations and a relatively small S.D. All statistical analyses were performed using the Prism5 software (GraphPad), and data plotting was carried out using SigmaPlot 11.0 (Systat Software, Inc.). In the bar graphs, columns represent the mean of all measurements with corresponding standard errors of the mean. Statistical significance for comparisons between different groups was established using either a Student’s t test or one-way analysis of variance followed by an all pairwise multiple comparison procedure (Bonferroni’s method), where ***, p ≥ 0.001; **, p < 0.01, and *, p < 0.05. In bioluminescence experiments, values of Firefly activity were normalized to the Renilla signal from the same well and then reported over the untreated values, stated as 100%. The different statistical tests are reported in the figure legends.

Bioinformatic Analysis of RNA Sequences—For bioinformatic analysis, BDNF 5' UTR sequences obtained from NCBI (www.ncbi.nlm.nih.gov) were analyzed using BioEdit nucleic acid annotation, and the number and position of uAUGs and uORFs were determined using Sequence Analysis Software. The mRNA free energy content was determined using the specific tool provided by the Vienna RNA Websuite free software package, a comprehensive collection of tools for folding, design, and analysis of RNA sequences (31).

RESULTS

Generation of a BDNF mRNA Variant Translatability Assay—To determine the role of the different 5' and 3' UTRs in modulating BDNF translation, we constructed a reporter plasmid, in which the Firefly luciferase (Fluc) gene, under the control of a cytomegalovirus promoter, was fused to each of the 10 exons coding for the 5' UTR (exons 1, 2a, 2b, 2c, 3, 4, 5, 6, 7, and 8) or, separately, with the 3' UTRlong or 3' UTRshort of rat bdnf (Fig. 1b). To evaluate the influence of the Kozak sequence on BDNF translation, the initial sequence of the Firefly luciferase gene was replaced by a stretch of nine nucleotides containing the AUG at the beginning of the canonical coding region in the rat exon 9 of the bdnf gene (K-9; Fig. 1, c and d). SH-SYSY neuroblastoma cells were co-transfected with a plasmid expressing the Renilla luciferase (Rluc) and the cytomegalovirus-bdnf 5' UTR-Fluc plasmids or the control Firefly luciferase (Fluc) plasmid. The amount of transfected Fluc plasmid mRNA...
showed slight differences for the different constructs as determined via semi-quantitative RT-PCR; therefore, luciferase data were normalized to the mRNA levels. Fig. 1c shows that in unstimulated SH-SYSY cells, each bdnf 5′UTR region determined a different translatability of the Fluc reporter gene, quantified by measuring luciferase bioluminescence (n = 10 cultures, Fig. 1c). Most 5′UTRs, as well as the canonical bdnf Kozak sequence (K-9), were able to significantly reduce the basal bioluminescence of the luciferase reporter gene. However, bdnf 5′UTR exons 3 and 5 had no effect on the basal Fluc translation, and exon 2a, coding for a short bdnf 5′UTR (205 nt), was able to strongly enhance the luciferase signal (Fig. 1c). Western blot analysis with an anti-luciferase antibody using SH-SYSY cell homogenates showed that the expressed luciferase protein had the expected molecular weight and supported the conclusion of a differential translatability of 5′UTR bdnf-Luc constructs (Fig. 1d).

Bioinformatic Analyses of bdnf 5′UTRs—The observed different translatability of 5′UTR bdnf variants prompted bioinformatic investigations to search for possible explanations for these findings. Intra-strand base pairing can produce secondary structures whose stability is quantified as the amount of free energy released or used by forming base pairs. Generally, an RNA molecule is folded in the most thermodynamically stable structure, the one having the minimum free energy (32), which means that the higher the free energy (more negative), the more stable and structured the RNA molecule (i.e. it is less accessible for the cap-dependent translation complex). Accordingly, using the RNA-fold package, we calculated the minimum free energy content in kilocalories/mol of the different bdnf 5′UTRs. The bar graph in Fig. 2a shows the free energy content of the different bdnf 5′UTR RNAs (red bars) in comparison with their translation rate measured with the dual-luciferase assay (black bars). The RNAs with the highest free energy (exon 1, 2c, 6, and 2b free energy < −150 cal/mol) correspond to the ones that strongly suppress the Fluc translation (Fig. 2a). On the contrary, exons 4, 8, 3, 7, 2a, and 5, which drive the translation of the reporter gene more efficiently, have a reduced free energy content (free energy > −100 cal/mol), which indicates a more relaxed and accessible RNA folding, thus resulting in a less severe repression of Fluc translation. Fig. 2 also shows that the computed free energy roughly correlates with the length of the 5′UTR region examined. For instance, exon 1, the longest bdnf 5′UTR variant with a length of 640 nt, has the highest free energy (−222 cal/mol; Fig. 2b), and exon 5, the shortest 5′UTR of only 81 nt, has the lowest free energy (−36 cal/mol; Fig. 2b).

Because basal mRNA translatability is influenced by several factors other than the free energy content, we also investigated for the presence of uORFs and the GC content, both features able to influence translation. It is known that upstream AUG trimucleotides (uAUGs) are apparently involved in down-regulation of translation (33). An AUG flanked by a purine (A/G) in position −3 and a G at position +4, relative to A in the start codon, acts as a proper driver of the translation process and is known as strong Kozak consensus sequence. Consequently, a weak Kozak has both positions differing from the consensus, whereas an adequate Kozak has only one discrepancy (34, 35). Noteworthy, exon 2a, which showed the highest translatability, has the lowest GC percentage (49.1%) and no upstream AUGs, whereas exons 1, 2c, 6, 4, and 8 have several AUGs (Fig. 2c). In detail, exon 1 has six uAUGs, of which three have an adequate consensus Kozak sequence and hence can act as moderate initiation sites, whereas the other three AUGs have a weak Kozak code (Fig. 2c). Exon 2c has only one adequate Kozak consensus sequence, whereas exons 6 and 4 have at least two adequate...
Kozak sequences each, and exon 8 bears two strong Kozak sequences along with six weak Kozak sequences. Finally, exon 3, which has relatively higher translatability with respect to the other bdnf 5’UTRs, bears only two weak Kozak consensus sequences (Fig. 2c). Bioinformatic analysis predicted that all these Kozak sequences can give rise to short uORFs (Fig. 2c; see horizontal bars below each exon) encoding short peptides with variable lengths and a different codon start (Fig. 2c; see +1, +2, or in-frame, below each exon).

**BDNF 5’UTRs Translational Regulation**—In the next series of experiments, the different bdnf splice variants were analyzed for their response to different receptor agonists in neuroblastoma SH-SY5Y cells (Figs. 3 and 4). 24 h after luciferase construct transfection, cells were treated for 3 h (n = 3 independent cultures) with KCl to induce a generalized depolarization or receptor agonists and neurotransmitters at the indicated concentrations as follows: KCl (50 mM); BDNF (50 ng/ml); glutamate (20 μM); DHPG (50 μM); AMPA (30 μM); NMDA (30 μM); ACh (30 μM); NE (50 μM); dopamine (40 μM); and 5-HT (50 μM). Each drug was tested in a viability assay, and because no appreciable cell death could be detected, we concluded that the concentration used was not toxic for the cells (Fig. 3a). Moreover, we also measured the luciferase luminescence response to these stimuli in comparison with basal levels (Fig. 3b). Once the robustness of the translation assay was assessed, the different drugs were tested systematically on each 5’UTR-bdnf luciferase construct (n = 3 independent cultures), and the luciferase levels of stimulated constructs were compared with the basal levels of the corresponding unstimulated constructs in the same experiment. In the majority of cases, no statistically significant effects on 5’UTR-bdnf translation levels were detected with some exceptions, including a significant increase in exon 1-driven translation following incubation with BDNF or 5-HT, and exon 7-driven translation with dopamine (Student’s t test; Fig. 4). In addition, we observed a significant reduction in exon 6-driven translation in response to BDNF, DHPG, AMPA, NMDA, and acetylcholine, and a reduction of exon 2b-driven translation following incubation with BDNF, dopamine (in this case also of exon 2a), and 5-HT (Student’s t test; p values as indicated in Fig. 4).

**BDNF CDS and 3’UTRshort and 3’UTRlong Translational Regulation**—Subsequently, basal and drug-induced translation of the luciferase reporter gene driven by BDNF short or long 3’UTR or the bdnf coding region sequences were measured (n = 3 independent cultures). In unstimulated conditions, bdnf CDS (cloned upstream to the luciferase gene), the short 3’UTR, and the long 3’UTR (cloned downstream to the luciferase) acted as translation repressors with respect to the basal luciferase gene (n = 10 independent cultures; Student’s t test p < 0.001), although the 3’UTRlong reduced translation more than the short 3’UTR (Fig. 5). However, neither the CDS nor the short or the long 3’UTR displayed any statistically significant response to drug stimulation (n = 3 independent cultures; Student’s t test; Fig. 5).

**Translational Regulation of Complete bdnf UTR Elements**—Given the poor responsiveness of the 5’UTR or 3’UTR or the CDS separately, we hypothesized that BDNF translation might require the simultaneous presence of the 5’UTR and 3’UTR to ensure a physiological regulation of translation. To test this hypothesis, we built constructs carrying the complete regulatory sequences present both in 5’ and 3’ untranslated regions for the four most abundant 5’UTR exons of bdnf, namely exons 1, 2c, 4, and 6. These 5’UTRs were individually inserted upstream to the luciferase gene that was followed by the long form of the 3’UTR. The complete constructs showed low levels of basal translation (n = 8 independent cultures, Fig. 6, basal). Neuronal depolarization by KCl, which can cause the extracellular release of endogenous neurotransmitters and trophic factors, induced a significant increase in translation of exons 1, 2c, and 4 with long 3’UTR (KCl; Student’s t test, p values as indicated in Fig. 6). Comparison of translatability of each complete construct in response to the different receptor agonists showed selective regulation. Exon 2c-Fluc-3’UTRlong was the only construct to be highly translated in response to BDNF, AMPA, and NMDA (n = 4 independent cultures; Student’s t test, Fig.
6). Exon 1-Fluc-3’UTRlong translation was enhanced by KCl, as mentioned above, and AMPA, but it was significantly reduced by acetylcholine, norepinephrine, and dopamine (*p < 0.05*). For all other panels, the luciferase activity values are expressed as fold over control values of luciferase from untreated cells (RLU). The exons tested are given in the x axes. Data shown (mean ± S.E.) are representative of three independent experiments performed in duplicate; dashed lines represent the control value (**, *p < 0.01; *, p < 0.05 with Student’s t test).

**Measurement of Antidepressants Effects on BDNF Translation**—Transcription of specific BDNF transcripts in rodents is known to be differentially regulated by different drugs, lengths of treatment, and drug/physical exercise combination, as well as stress paradigms (37–39). Donnici et al. (27) studied the effect of different antidepressants on BDNF mRNA expression in human neuroblastoma SH-SY5Y cells, showing biphasic changes in the expression of total and specific BDNF transcripts in human cells following antidepressant treatments. However, no data were available on the effect of antidepressants on BDNF transcript translation in vitro. Because many antidepressants are active on both the noradrenergic and the serotonergic systems, we first investigated the effects of a combined stimulation with serotonin and norepinephrine (Fig. 7, 5-HT+NE). The combination of 5-HT and norepinephrine caused a significant increase in translation of Exon 2c-Fluc-3’UTRlong and Exon 6-Fluc-3’UTRlong transcripts (*p < 0.05*). We then tested whether stimulation with the two antidepressants desipramine and mirtazapine, known to increase both serotonin and norepinephrine levels, was able to affect translation. Desipramine is a tricyclic antidepressant (TCA) that inhibits the reuptake of norepinephrine and to a lesser extent serotonin; mirtazapine is a tetracyclic piperazinoazepine antidepressant, which has a different structure from any other currently used antidepressant. It enhances central noradrenergic and serotonergic activity by blocking α2 receptors and selectively antagonizing 5-HT2 and 5-HT3 receptors. Thus, it is being classified as a noradrenergic and specific serotonergic antidepressant and is referred to as an NaSSA. Desipramine stimulation for 3 h, i.e. for the same time as for stimulation with the receptor agonist, induced significant up-regulation of Exon 2c-Fluc-3’UTRlong (*p = 0.007*, Student’s t test; *n = 4* independent cultures, Fig. 7, Desipra-
DISCUSSION

We show here that bdnf transcripts have different basal translatability and pharmacological profile of translation in response to agonists of the main excitatory neurotransmitter receptors and BDNF itself. Moreover, we show that neurotransmitter- or BDNF-induced translation of bdnf mRNA requires the presence of both the 5'UTR and the 3'UTR sequence as these regions or the coding sequence alone are unable to modulate translation of a reporter gene. On the basis of these results, we propose that bdnf mRNA variants represent a "quantitative code" to modulate the expression of the protein in response to specific stimuli.

We have developed a cell-based luciferase reporter assay able to measure the translatability of BDNF variants under basal conditions or in response to a drug treatment. This translation assay allows assessment of drug effects on translation of each bdnf variant in a small volume, scalable setup from 96- to 384-well plates or more. This cell-based assay is robust, high throughput, and quantitative because it is based on luciferase bioluminescence that displays an extensive dynamic range of responsiveness and provides reproducible results. In addition, thanks to the fact that it was developed in undifferentiated cells, this assay can be run as early as 24 h from plating cells. Currently available drug screening assays for modulators of BDNF levels are based on measurement of BDNF exon 2 in a cellular model of Huntington disease (patent US20050123922 (41)) or on measurement of the transcription of four fish bdnf mRNA variants in wild-type zebrafish embryos (patent US7615676B2 (42)). However, both methods can screen for modulators of bdnf transcription but do not allow one to determine the expression of all possible BDNF variants and do not obtain information on the final amounts of the BDNF protein produced in response to the drugs. Similarly, YAC or BAC transgenic mouse lines were used to monitor mRNA expression (14–16). However, it is very well known that the levels of the mRNA encoding BDNF do not correspond to the actual BDNF protein levels in vivo (43).

The results obtained with our cell-based BDNF translation assay indicate that, in human SH-SY5Y cells, each bdnf 5'UTR region determines a different basal translation level of the reporter gene, with most 5'UTRs acting as translation repres-
Translational Regulation of BDNF Variants

The translation ability of an mRNA resides in its UTR regions, particularly in the one located at the 5′ end. The presence of a 5′ cap, secondary structures, multiple ORFs, multiple upstream AUGs (uAUGs) in different Kozak contexts, internal ribosome entry sites, and the binding of trans-acting factors is relevant for translational regulation. All these features can control the synthesis of a protein by affecting stability of the mRNA, its accessibility to ribosomes, circularization, and interaction with the translation machinery. Through a bioinformatics approach, we identified sequence features in the 5′ UTR exons that could be responsible for the differential expression of the various bdnf isoforms observed in the reporter assay. In particular, we found that the different 5′ UTRs have a different length, GC content, and a different number of uAUGs (up to six). Interestingly, all these AUGs, no matter if in a strong or weak Kozak context, can potentially produce short peptides each representing a uORF, with variable lengths and a different codon start. Whether these short peptides are indeed produced and what is their function, it is currently unknown and may be worthy of further investigations.

There are several examples of specific alterations in expression levels of BDNF variants in various neurological disorders or traumatic conditions in human post-mortem samples that have been replicated in animal models (46–49). A crucial question is what potential use can have drugs able to stimulate selective translation of single bdnf mRNA variants. The answer is...
obvious when only one or two variants are affected and when one receptor agonist can specifically up-regulate these isoforms. In more complex situations, we believe that a possible answer to this question may reside in our previously proposed “spatial code” model of BDNF splice variants (50–52). According to this model, BDNF mRNA variants become spatially segregated in response to neuronal activation and acquire a localization within three distinct subcellular domains as follows: the soma (exons 1, 3, 5, 7, 8, and 9a), the proximal (exon 4), or the distal (exon 2 and 6) dendrites (36, 53). Of note, each of these dendrites (36, 53). Importantly, the BDNF protein generated from each individual transcript segregates to the same subcellular domain of the transcript that has generated it, leading to a remarkably localized secretion of BDNF and activation of TrkB within the same dendritic domain (53, 54). This localized activation of the BDNF/TrkB signaling cascade has functional consequences because it results in marked changes in dendritic and spine morphology only in the compartment interested (53, 54).

Morphological changes occurring at restricted cellular regions are known to affect the balance between excitatory (glutamatergic), inhibitory (GABA and glycinergic), and modulatory (5-HT, NE, ACh, and dopamine) levels because these types of synapses are generally segregated from each other in most brain regions. For instance, pyramidal neurons of cortical layer 5 in the visual cortex as well as hippocampal neurons receive segregated synaptic inputs from GABAergic fibers on the cell soma and proximal dendrites, whereas glutamatergic inputs are mostly found on distal dendrites (55, 56). Accordingly, segregated BDNF transcripts may be selectively recruited to modulate different types of synaptic contacts. Indeed, mice with a selective ablation of exon 4 transcripts (localized in proximal dendrites) were shown to exhibit significant deficits in GABAergic interneurons in the prefrontal cortex, particularly those expressing parvalbumin, a subtype implicated in executive function and schizophrenia (57). Moreover, disruption of promoter 4-driven bdnf transcription impaired inhibitory but not excitatory synaptic transmission recorded from layer 5 pyramidal neurons in the prefrontal cortex (57). One example of how BDNF mRNA segregation may impact the neuronal connectivity is given by exon 2c, which was the only construct to be highly translated in response to BDNF, AMPA, and NMDA. This transcript is specifically translated in response to agonists present at excitatory glutamatergic synapses known to induce long term potentiation, but it does not respond to the metabotropic glutamate receptor agonist DHPG known to induce long term depression.

The mechanisms regulating translation of BDNF splice variants in neurons are still unknown. However, this study provides the first fundamental information regarding the neurotransmitters that can activate translation of the different BDNF transcripts. In particular, translation of transcripts containing exons 2 and 6 and the 3’UTRlong is strongly up-regulated when serotonin and norepinephrine are given together, a condition that is typically achieved by several antidepressants. Together with the fact that translation of exon 6–3’UTRlong transcript is strongly enhanced by dopamine, and exon 2c-3’UTRlong by BDNF and AMPA, and both are activated by KCl, it is conceivable that to be translated exons 2 and 6 transcripts may require synergistic activation of more signaling cascades. Of note, exons 2 and 6 mRNAs are localized to distal dendrites and are locally translated in response to KCl (53). Interestingly, serotonin was previously described to activate translation in Aplysia through a biochemical pathway involving protein kinase C, cyclic AMP-dependent protein kinase, and could be inhibited by rapamycin, indicating involvement of the mammalian target of rapamycin pathway for regulation of protein translation (58). Moreover, it has recently been shown that activation of D1 dopamine receptors also activates protein translation through the same pathway resulting in activation of mTORC1, increased phosphorylation of ribosomal protein S6, and eukaryotic translation initiation factor 4E-binding protein (59). In our study, individual agonists did not have an effect on translation of exon 1 and 4 transcripts (or, as in the case of dopamine, cause a decrease). In contrast, KCl depolarization was able to enhance translation of exon 1 and 4 transcripts suggesting that release of several neurotransmitters and growth factors at the same time may be required to trigger translation of these transcripts. Indeed, it has been shown that activation of the ERK pathway by D1 dop-

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**FIGURE 7. 5’UTR-Firefly luciferase-3’UTR BDNF-induced translation.** SH-SYSY cells were transfected with plasmids containing 5’UTR-luciferase-3’UTR BDNF constructs and treated for 3 h with combined 5-HT (50 μM) and NE (50 μM) or the antidepressants desipramine (10 μM) or mirtazapine (50 μM). The luciferase activity values are expressed as fold over control values of luciferase from untreated cells (RLU). The exons tested are given in the x axes. Data shown (mean ± S.E.) are representative of four independent experiments performed in duplicate; dashed lines represent the control value (**, p < 0.01; *, p = 0.05 with Student’s t test).
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amine receptor requires activation of glutamatergic transmission (60).

In conclusion, we set up a cell-based assay to screen for natural or synthetic compounds able to treat neurological diseases through an increase or decrease of BDNF protein levels produced by translation of the different BDNF splice variants. It is composed of vectors for protein expression in eukaryotic cells of the various exons of the BDNF gene encoding the 5’- and 3’UTRs in fusion with a reporter gene. We expect that future research and drug screening assays will need to be focused on pharmacological modulation of individual transcripts of BDNF; in this context, our tools may help with designing novel and more specific treatments for neuropsychiatric diseases.

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