HHS Public Access

Author manuscript

SLAS Discov. Author manuscript; available in PMC 2024 January 02.

Published in final edited form as:

SLAS Discov. 2023 April ; 28(3): 88–94. doi:10.1016/j.slasd.2023.02.005.

High throughput assay for compounds that boost BDNF expression in neurons

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Abstract

Deficiencies in brain-derived neurotrophic factor (BDNF) have been linked to several brain disorders, making compounds that can boost neuronal BDNF synthesis attractive as potential therapeutics. However, a sensitive and quantitative BDNF assay for high-throughput screening (HTS) is still missing. Here we report the generation of a new mouse *Bdnf* allele, $Bdnf^{NLuc}$, in which the sequence encoding nano luciferase (NLuc) is inserted into the *Bdnf* locus immediately before the stop codon so that the allele will produce a BDNF-NLuc fusion protein. BDNF-NLuc protein appears to function like BDNF as $Bdnf^{NLuc/NLuc}$ homozygous mice grew and behaved almost normally. We were able to establish and optimize cultures of cortical and hippocampal $Bdnf^{NLuc/+}$ neurons isolated from mouse embryos in 384-well plates. We used the cultures as a phenotypic assay to detect the ability of 10 mM KCl to stimulate BDNF synthesis and achieved a reproducible Z′ factor > 0.50 for the assay, a measure considered suitable for HTS. We successfully scaled up the assay to screen the 1280-compound LOPAC library (Library of Pharmacologically Active Compounds). The screen identified several BDNF-boosting compounds, one of which is Bay K8644, a L-type voltage-gated calcium channel (L-VGCC) agonist, which was previously shown to stimulate BDNF synthesis. These results indicate that our phenotypic neuronal assay is ready for HTS to identify novel BDNF-boosting compounds.

Keywords

brain-derived neurotrophic factor; nano luciferase; knockin; gene expression; HTS assay; LOPAC library

Introduction

Brain-derived neurotrophic factor (BDNF), a 28-kD dimeric secreted growth factor, binds to TrkB receptor tyrosine kinase, inducing its dimerization and activation [1]. This

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activates many signaling cascades that cooperatively promote neuronal survival, regulate the development of neural circuits, stimulate synapse formation, enhance synaptic transmission, and facilitate synaptic plasticity in many brain regions [2–4]. These crucial physiological functions implicate BDNF deficiency as a potential cause for several neuropsychiatric and neurodegenerative disorders, making restoring/elevating BDNF function an attractive therapeutic goal [5–11].

The *Bdnf* gene is transcribed from 9 distinct promoters, each of which drives transcription of a short 5′ exon alternatively spliced onto a common 3′ exon (Exon 10) encoding the BDNF protein [12, 13]. Furthermore, *Bdnf* mRNAs transcribed from each promoter are polyadenylated at two alternative sites, leading to two distinct populations of mRNAs: those with a short 3′ untranslated region (UTR) and those with a long 3′ UTR [14, 15]. Thus, BDNF synthesis is regulated at levels of transcription and translation [14, 16, 17].

Three approaches have been employed to increase BDNF-TrkB signaling in the CNS: recombinant BDNF [18, 19], small molecule or antibody TrkB agonists [20–25], and viral expression of BDNF [26–28]. Each approach has limitations. First, BDNF, as a protein, is limited by short half-life, poor blood-brain barrier (BBB) penetration, and poor brain intraparenchymal penetration [29, 30]. Accordingly, recombinant BDNF failed clinically for treating amyotrophic lateral sclerosis [18, 19]. Second, it is very challenging to find small molecule (<MW 500) TrkB agonists, as they must induce TrkB dimerization. In support, more rigorous studies demonstrated that previously identified small molecule TrkB agonists neither bind nor activate TrkB [20, 21, 31]. While monoclonal antibodies may succeed [32], few can cross the BBB. In addition, neither small molecule nor antibody TrkB agonists can achieve the synapse-specific and time-sensitive effects of endogenous BDNF [33–36]. Lastly, using viral vectors to express BDNF in the brain is very invasive. As a progressing disease spreads to multiple brain regions, multiple injections of virus would be necessary to produce adequate BDNF. Furthermore, the safety of such protocols is dubious, with no good way to turn off viral BDNF expression in case of side effects.

We explored a more challenging, technically feasible, yet-under-explored approach: to screen for compounds that can stimulate BDNF expression in neurons, by taking advantage of highly active nano luciferase (NLuc), which is ~150-fold more active than firefly luciferase [37]. This approach is phenotypic, as compounds operating by any relevant operable mechanism may be uncovered, yet effects would be focused on BDNF enhancement. Here, we report establishment of an NLuc-based HTS assay for BDNF-boosting compounds.

Materials and Methods

Animals.

Wild-type C57BL/6J mice were purchased from the Jackson Laboratory. All mice were given free access to food and water and housed in a 12 h/12 h light/dark cycle. All procedures described here were approved by the Institutional Animal Care and Use Committee at UF Scripps Biomedical Research and were in compliance with the National Institutes of Health guide for the care and use of laboratory animals.

Generation of Bdnf^{NLuc} allele.

We generated a *Bdnf*^{NLuc} mouse allele by inserting the NLuc-coding sequence immediately before the stop codon at the *Bdnf* locus using the CRISPR/Cas9 technique. sgRNAs were designed using the CRISPR tool (http://crispr.mit.edu) to minimize potential off-target effects. Two sgRNAs were selected (sgRNA1: 5'- GTGTATGTACACTGACCATT-3'; sgRNA2: 5'-GAATTGGCTGGCGATTCATA-3') for integration of the NLuc sequence. The DNA donor plasmid contains two homology arms flanking the NLuc sequence, which were cloned into BamHI digested pBluescript II KS (−) vector using the Gibson assembly method (NEB, E5510). The 5' homology arm contains the 850-bp sequence upstream of the stop codon, whereas the 3' homology arm contains the 803-bp sequence downstream of the stop codon. To block further Cas9 targeting and recutting after undergoing homology-direct repair, we introduced silent mutations into the PAM motifs of the two sgRNAs located within the 5' homology arm in the donor plasmid by using Q5 Site-Directed Mutagenesis kit (NEB, E0054). The donor plasmid was confirmed with DNA sequencing. Microinjection of mixture of sgRNAs, donor DNA, and Cas9 protein was performed by Genomic Modification Facility at Scripps Research. Zygotes were cultured to the blastocyst stage in vitro. Genomic DNA from blastocysts was extracted and PCR screen was performed to select blastocysts with successful homologous recombination. Positive blastocysts were transferred into the oviducts of pseudo-pregnant females to produce the founder mice.

Primary cortical/hippocampal cell culture and maintenance.

Brains of E18.5 *Bdnf*$^{NLuc/+}$ embryos were collected and placed in ice-cold Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS, Gibco #14185–052) supplemented with Penicillin-Streptomycin (Pen/Strep, 50 U/mL, Gibco #15140–122), 25 mM D-glucose (Sigma #G8270), 1 mM pyruvate (Gibco #11360–070), and 20 mM HEPES (Gibco #15630–080). Cortices and hippocampi were isolated and digested with papain (~12 U/mL, Worthington #LS003126) at 37°C for 20 min. Brain tissues were washed with media containing Neurobasal (Gibco #21103–049), 4% fetal bovine serum (FBS, HyClone #SH30088.03), and Pen/Strep. The tissue was gently triturated and allowed to settle, and the supernatant was filtered through a 40-μm cell strainer. Cells were centrifuged at 420 xg for 4 min and resuspended in plating media [Neurobasal, 2% FBS, 2% B27 (Gibco #17504–044), 2 mM GlutaMAX (Gibco #35050–061), and Pen/Strep]. 1.0×10^4 or 4.5×10^4 cells were plated onto white Greiner μClear 384-well plate (80 μl/well) or PDL-coated 96-well plate (200 μl/well), respectively. Sixteen hours after plating, 75% of the plating media was replaced by feeding media [Neurobasal, 2% B27, 2 mM GlutaMAX, and Pen/Strep] supplemented with 16 μM 5-fluoro-2′-deoxyuridine (FUdR, Sigma #F0503) to prevent overgrowth of non-neuronal cells. Plating and media changes were performed using a Versette liquid handler (Thermo Fisher Scientific) or an 8-channel pipette for the 384-well and 96-well plates, respectively. Cells were maintained at 37°C and 5% CO_2 incubator.

Compound treatment and LOPAC library screening.

Compound treatment or library screening in primary cultures of *Bdnf*$^{NLuc/+}$ mouse neurons on day 7 in vitro (DIV7) was performed by replacing one-quarter of the culture medium with equal volume of compound-containing medium to yield the desired final concentration

for each compound. For the library of pharmacologically active compounds (LOPAC, Millipore #LO3300), compounds in the original source plates were first dosed using a pintool into 384-well plates containing feeding medium to create a secondary source plate with compounds at 40 μM using the Beckman Coulter's Biomek NX^P automated liquid handler (Beckman). 1.6% DMSO and 40 μM (±)-Bay K8644 (Sigma #B112) were added to the first two and the last two columns of the secondary source plate as vehicle and positive control, respectively. Compounds in the secondary source plates were then added to culture neurons in the 384-well plates using Versette liquid handler. The final concentration of compound and DMSO in each well is 10 μM and 0.4%, respectively. Neurons were treated with compounds for 24 h in a humidified incubator set to 5% CO_2 and 37°C. Luciferase activity was detected the next day using Nano-Glo Luciferase Assay System (Promega #N1150) according to the manufacturer's instruction. Luminescence intensity was measured using microplate reader (FLUOstar Omega, BMG LABTECH) at 460 nm.

Immunocytochemisty.

Primary cultures of *Bdnf*$^{NLuc/+}$ mouse neurons were plated onto PDL-coated 15 mm coverslips (Chemglass, Life Sciences #CLS-3500–012) at the density of 8×10^5 cells per well in 12-well plates. On DIV7, neurons were fixed with 4% paraformaldehyde (Sigma #441244) supplemented with 120 mM sucrose (Sigma #S0389) in phosphate buffered saline (PBS) for 20 min and permeabilized with 0.25% Triton X-100 (Sigma #T8787) in PBS for 10 min at room temperature (RT). Neurons were then incubated with blocking buffer [0.1% Triton X-100, 10% bovine serum albumin (BSA, Sigma #BP1600–1) in PBS] for 1 hour at RT, and then incubated with primary antibody diluted in dilution buffer (0.1% Triton X-100, 1% BSA in PBS) for 2 hours at RT or overnight in 4°C. The following primary antibodies (Millipore) were used: NeuN (1:1,000; #ABN78), GFAP (1:500; #MAB360), MAP-2 (1:500; #MAB3418), PSD-95 (1:1,000; #MABN68), and synaptophysin (1:1,000; #MAB5258). After three washes in PBS, neurons were incubated with appropriate secondary antibodies (1:500; Jackson ImmunoResearch) for 1 hour at RT, washed three times with PBS, and mounted to slides using DAPI Fluoromount-G medium (SouthernBiotech #0100–20). Images were acquired using a Nikon C2+ confocal microscope.

Protein extraction and luciferase activity measurement.

Cortices and striata of 10-week-old wild-type (WT) or *Bdnf*$^{NLuc/NLuc}$ mice were isolated and homogenized using RIPA lysis buffer supplemented with protease inhibitors (cOmplete Mini, Sigma #11836153001). Protein extracts were obtained after lysates were centrifuged at 17,922 xg (13,200 rpm in Eppendorf 5417R) for 20 min at 4°C. Protein concentration was determined with BCA assay kit (Thermo Fisher Scientific #23227). Luciferase activity in protein extracts was detected using Nano-Glo Luciferase Assay System (Promega). Luminescence intensity was measured using microplate reader (FLUOstar Omega, BMG LABTECH) at 460 nm.

Results

Generation of *Bdnf*^{NLuc} knockin mice

BDNF levels in tissues or cultured neurons are currently measured with Western blot or ELISA, detection methods too slow and expensive for high throughput screening (HTS). We reasoned that NLuc could allow us to develop a sensitive and quantitative assay for BDNF detection that is HTS amenable and cost effective. We employed the CRISPR/Cas9 technology to generate a *Bdnf*^{NLuc} allele (Fig. 1A). We obtained 13 mice from injection of two sgRNAs and one donor plasmid. Using PCR, we identified 5 mice containing the expected NLuc insertion at the *Bdnf* locus (Fig. 1B). Of the 5 mice, two mice produced *Bdnf*$^{NLuc/+}$ offspring after they were crossed to WT mice. As expected, *Bdnf*$^{NLuc/+}$ mice produced both *Bdnf* mRNA and *Bdnf-NLuc* mRNA (Fig. 1C). Also, *Bdnf-NLuc* mRNA was detected in the cortex of *Bdnf*$^{NLuc/+}$ mice but not in the WT cortex or in the *Bdnf*$^{NLuc/+}$ striatum where the *Bdnf* gene is not expressed [38] (Fig. 1D). These RT-PCR results indicate that the *Bdnf*^{NLuc} allele is expressed and regulated in the same way as a normal *Bdnf* allele. We detected NLuc activity in cortical lysates of *Bdnf*$^{NLuc/NLuc}$ mice using Promega Nano-Glo Luciferase Assay System (Fig. 1E). We also detected NLuc in the striatal lysates of *Bdnf*$^{NLuc/NLuc}$ mice (Fig. 1E), indicating that BDNF-NLuc is transported to the striatum from BDNF-expressing afferent neurons as does BDNF. No NLuc activity was detected in cortical and striatal lysates of WT mice (Fig. 1E).

Bdnf$^{NLuc/NLuc}$ mice grew normally, did not show aggression like *Bdnf*$^{+/-}$ heterozygous mice [39], were fertile, and produced normal size of litters. However, *Bdnf*$^{NLuc/NLuc}$ mice became modestly heavier than WT mice (32.1 ± 1.9 g vs. 27.0 ± 0.7 g for male mice at 11 weeks old, p=0.033; 22.5 ± 0.5 g vs. 20.0 ± 0.2 g for female mice at 10 weeks old, p=0.014). These phenotypes indicate that the BDNF-NLuc fusion protein functions nearly as efficiently as BDNF in the nervous system, which is likely owing to the small size of NLuc (19.1 kDa).

Culturing *Bdnf*$^{NLuc/+}$ neurons in 384-well microplates

The first step toward HTS assay development is to set up neuronal cultures in a miniaturized format. We could establish cultures of neurons isolated from cortices and hippocampi of newborn *Bdnf*$^{NLuc/+}$ pups in both 96- and 384-well plates (Fig. 2A, B). We selected the cortex and hippocampus as the source of primary neurons for their relevance to many CNS disorders and their richness in BDNF-expressing neurons. We could obtain approximate 1×10^7 cells from the cortex and hippocampus of one newborn pup, which is sufficient to seed a 96-well plate (~50,000 cells/well) or a 384-well plate (~20,000 cells/well). The assay for NLuc activity in cultured *Bdnf*$^{NLuc/+}$ neurons was highly specific, producing a signal-to-noise ratio (S/N) of 1406, because neurons did not emit any luminescence in the absence of the NLuc substrate furimazine (Fig. 2A). The assay was also highly sensitive, as *Bdnf*$^{NLuc/+}$ neurons cultured in 384-well plates still produced robust luminescence (Fig. 2C). As BDNF is a secreted protein, BDNF-NLuc was detected in both cells and media (Fig. 2C). However, as indicated by large CVs (coefficients of variation; Fig. 2C), there was a large well-to-well variation in NLuc activity and thus the assay was not suitable for compound screening. In addition, the viability of the culture was not consistent, and neurons

in some cultures died during the first few days. These issues may be in part attributable to the postnatal nature of isolated neurons.

Optimization of *Bdnf*$^{NLuc/+}$ neuronal assay for HTS

To solve the issue of large variation, we optimized the assay by changing culture conditions. After persistent efforts, we have established a procedure to greatly improve assay reliability and reproducibility. The procedure includes culturing *Bdnf*$^{NLuc/+}$ neurons isolated from cortices and hippocampi of embryos (from WT x *Bdnf*$^{NLuc/NLuc}$ crosses) at embryonic day 18.5, using a liquid handling device to plate cells and handle other solutions in 384-well plates, plating 10,000 cells/well, plating cells with a Neurobasal medium containing 2% fetal bovine serum and 2% B27 and then replacing 75% of the plating medium with a Neurobasal medium containing 2% B27 a day later, and adding the thymidylate synthase inhibitor FUDR to media to kill rapidly dividing cells.

Using this new procedure, we could consistently establish healthy cultures of *Bdnf*$^{NLuc/+}$ neurons. To find an age when the *Bdnf*$^{NLuc/+}$ neuronal culture is suitable for compound screening, we measured the NLuc activity in media from cultures on DIV3, DIV6, DIV8, DIV10, and DIV12 (Fig. 3A). Well-to-well variation in NLuc activity in cultures on DIV6-DIV10 was small with a CV of ~5%. Furthermore, cells in *Bdnf*$^{NLuc/+}$ cultures on DIV7 were mostly neurons (Fig. 3B) with long dendrites (Fig. 3D) and many synapses (Fig. 3E), and only a small number of them were astrocytes (Fig. 3C). These results indicate that we can treat *Bdnf*$^{NLuc/+}$ cultures on DIV7 to determine if a compound has potentials to increase BDNF expression in neurons.

We tested our assay for suitability for HTS by using two positive controls. Several chemicals have been reported to stimulate *Bdnf* gene expression, including KCl [14, 40] and insecticide deltamethrin (DM, Sigma #253300) [41]. Membrane depolarization induced by incubation with KCl at 50 mM for 90 min increases *Bdnf* gene expression in cultured neurons for a short duration [14, 40]. We found that KCl at a final concentration of 10 mM elevated levels of BDNF in both cell bodies and media in our neuronal cultures over 24 hours without detectable cytotoxicity (Fig. 4A). DM also stimulated BDNF production in our neuronal cultures in a dose-dependent manner (Fig. 4B, C). The assay produces a good Z′ for both 10 mM KCl and 10 μM DM (Fig. 4A–C) [42]. Future HTS experiments should focus on NLuc activities in cells, because the cell assay has a slightly better Z′ (Fig. 4A–C) and saves time and cost in comparison with the medium assay. To measure NLuc activities in cells, we can simply invert a plate to dump culture medium and then add the Promega Nano-Glo mix, whereas we must transfer a certain amount of culture medium to a new plate and then add the Promega Nano-Glo mix to measure NLuc activities in media.

To determine if our assay has reproducibility throughout whole plate, we treated a half plate of neuronal culture with vehicle and the other half with 10 mM KCl for 24 hours, and the assay produced a Z' of 0.67 (Fig. 4D). The Z′ for DM is consistently lower than the one for KCl (Fig. 4), likely because DM is a less potent inducer of BDNF expression. Our NLuc assay in cells for 10 mM KCl has a reproducible Z′ > 0.50 (Fig. 4), a measure considered suitable for HTS [42].

Scalability and repeatability of the *Bdnf*$^{NLuc/+}$ phenotypic assay

We tested the scalability of the *Bdnf*$^{NLuc/+}$ phenotypic assay by screening the 1280-compound Library Of Pharmacologically Active Compounds (LOPAC). We dissected out cortices and hippocampi of 8 *Bdnf*$^{NLuc/+}$ embryos at E18.5 from 2 timed pregnant mice, prepared a suspension of dissociated neurons (10,000 cells/80 μl), transferred 40 ml of the suspension into each of eight 50-ml Falcon centrifuge tubes, used the liquid handling device to plate cells from each aliquot of 40-ml cell suspension onto a 384-well plate (80 μl/well), and cultured neurons. We removed 20 μl of medium from each well and then added each LOPAC compound in 20 μl of fresh medium to a well in 2 sets of 4 plates of neuronal cultures on DIV7 to reach a final concentration of 10 μM. LOPAC compounds (320) were added to wells in the middle 20 columns of each plate, while the first 2 and last 2 columns were used for vehicle controls and positive controls. After 24 hours of incubation, we inverted plates to dump culture medium and then measured NLuc activity in neurons. The NLuc activity in each well is normalized to the average of NLuc activities in 1280 compound-containing wells. The intra-assay CV between 2 sets of plates is 5.9% and the 8 plates have an average Z factor of 0.61 ± 0.04 for positive control Bay K8644, indicating excellent plate-to-plate assay repeatability. If a compound that increases the NLuc activity to more than 3x standard deviations (3xSDs) over the mean, it is considered a hit, and in this case, there are 7 hits in the 1st set of plates (Fig. 5A) and 10 hits in the 2nd set of plates (Fig. 5B), 7 of which are shared. When the average of duplicate assays for each compound is plotted, we identify the same hits from the 2nd set of plates except hit 8 (Fig. 5C). To test the repeatability of the assay in different batches of cultured neurons, we repeated the screening with 4 plates of cultured neurons. The repeat screening rediscovered all hits that were found in the original screening (hits 1, 2, 3, 4, 5, 7, and 10) except hits 6 and 9 (Fig. 5D; hit 9 is just under the threshold). To further test the reproducibility of the assay, we examined Bay K8644 in 3 batches of cultured neurons prepared on different weeks and found that the compound increased BDNF levels to a similar extent in the 3 trials (Fig. 5E). These results demonstrate high scalability and repeatability of the assay.

The hits include brefeldin A, L-type voltage-gated calcium channel (L-VGCC) agonist (Bay K8644), L-VGCC blockers (nitrendipine, nimodipine, N-propargyl nitrendipine, & felodipine), estrogen receptor alpha agonist [(1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole] (PPT), 5-HT1B and 5-HT1D antagonist (GR127935 hydrochloride hydrate), protein kinase C activator (phorbol-12-myristate 13-acetate), and cyclooxygenase inhibitor (phenylbutazone). Some of these hits are supported by previous reports. Brefeldin A was shown to inhibit protein transport from the ER to the Golgi complex [43], so that it can increase intracellular BDNF levels by inhibiting BDNF secretion. KCl stimulates *Bdnf* gene expression by activating Ca^{2+} influx through L-VGCC [14], and thereby Bay K8644 stimulates *Bdnf* gene expression in neurons by opening L-VGCC [44]. Phorbol myristate acetate was reported to enhance the effect of Bay K8644 on *Bdnf* gene expression [44].

Taken together, our screening of LOPAC compounds demonstrates that the *Bdnf*$^{NLuc/+}$ neuronal assay can be scaled up and that the assay can find hits that increase intracellular BDNF levels in neurons. Therefore, our assay using *Bdnf*$^{NLuc/+}$ neurons is suitable for a large-scale screening campaign.

Discussion

We have developed a novel and sensitive phenotypic assay to screen for BDNF-elevating compounds. We chose a phenotypic assay for two reasons. First, phenotypic drug discovery (PDD) is very effective for identifying first-in-class drugs compared to target-based drug discovery [45, 46]. Second, PDD is particularly powerful to address the complexity of diseases that are poorly understood (e.g., major depressive disorder and sporadic Alzheimer's disease) [47]. This assay has advantages of speed, cost, and throughput over measuring BDNF levels in a traditional manner (e.g., immunoblotting and ELISA). *Bdnf*$^{NLuc/+}$ neurons are much more powerful reagents in drug discovery compared to neurons from BAC BDNF-FLuc (firefly luciferase) transgenic mice, which have been used to screen 120 herbal extracts for inducers of *Bdnf* gene expression [48]. Because the BDNF-FLuc transgene expresses FLuc in the place of BDNF, the assay using BDNF-FLuc neurons only reports altered *Bdnf* transcription. In contrast, the *Bdnf*^{Nluc} allele expresses BDNF-NLuc fusion protein, so that our assay using *Bdnf*$^{NLuc/+}$ neurons can report changes in BDNF levels due to altered transcription, translation, stability, and/or secretion. Furthermore, NLuc is much more active than FLuc, which makes it possible to reduce the cell number per well, saving time and money in a large-scale screening campaign in a miniaturized format.

Using the *Bdnf*$^{NLuc/+}$ neuronal assay, a researcher can comfortably screen 1600 compounds in 5 sets of 384-well plates in duplicate every two weeks and thereby over 40,000 compounds in a year. The screen will identify hits that increase intracellular BDNF levels by stimulating BDNF expression via either transcription or translation, inhibiting BDNF secretion, or stabilizing BDNF. The hits that inhibit BDNF secretion or stabilize BDNF would not increase BDNF levels in media and could be eliminated through measurements of BDNF levels in both cells and media. To determine whether a hit selectively increases BDNF expression by activating a signaling cascade or a transcription factor or non-selectively increases BDNF expressing by enhancing overall transcription or translation, a counter assay is necessary. Such a counter assay can be established using the *Gt(ROSA)26Sor*$^{tm1(Luc)Kael}$/J mouse strain that expresses firefly luciferase (FLuc) in a Cre-dependent manner [49]. This strain of mice are crossed to mice that express Cre in germline cells to remove the transcription stop sequence in the lox-stop-lox cassette, creating a *Rosa26*^{FLuc} allele which expresses FLuc from the *Gt(ROSA)26Sor* locus in every cell. Because the *Rosa26*^{FLuc} allele is active in every cell of the body [50], the regulation of gene expression would be very different in *Rosa26*^{FLuc} and *Bdnf*^{NLuc}. Thus, *Rosa26*$^{FLuc/+}$ neuronal culture is a suitable counter assay to eliminate non-selective hits.

Deficiencies in BDNF-TrkB signaling have been linked to several neurological and psychiatric disorders, including Alzheimer's disease (AD), Huntington's disease (HD), major depressive disorder (MDD), anxiety, eating disorders, and Rett syndrome [5, 51–56]. A few compounds such as ampakines [57] and fingolimod [58] weakly boost BDNF synthesis and yet improve disease symptoms in mouse models for AD [59], HD [60, 61], and Rett syndrome [58, 62], supporting the strategy of using BDNF-boosting drugs for treatment of brain disorders associated with deficits in BDNF-TrkB signaling. A specific HTS campaign designed to discover compounds that more effectively boost BDNF synthesis using the assay described

here should deliver more useful leads for development as therapeutic reagents for the brain disorders.

Due to multiple promoters and alternative polyadenylation, the *Bdnf* gene produces 18 mRNA variants encoding the same BDNF protein [12, 13, 63, 64]. Distinct combinations of *Bdnf* mRNA variants are expressed in different tissues or brain regions [12, 63]. As distinct signaling pathways regulate production and translation of different *Bdnf* mRNA variants [14, 15, 17], an HTS using the $Bdnf^{NLuc/+}$ neuronal assay could find compounds that boost BDNF synthesis in selective brain regions, an aspect that may minimize undesired side effects. For instance, a compound that boosts BDNF synthesis in the cortex and hippocampus could prove useful for treating MDD, because it may not affect BDNF synthesis in midbrain dopaminergic neurons where BDNF has depressive effects [65, 66]. The usage of various *BDNF* promoters is very different in the brain and peripheral tissues in humans [63]. For example, transcripts from promoters 1, 2, 4 and 5 are abundant in the brain, but low or absent in peripheral tissues [63]. Therefore, it is possible to find compounds that boost BDNF production in the brain without causing the sensation of pain that is associated with the activation of BDNF receptors in nociceptors [67]. TrkB agonists do not offer such benefits.

ACKNOWLEDGEMENTS

We thank Dr. Thomas Bannister for editing the manuscript. This work was supported by the grants from the National Institutes of Health to BX (R01 DK103335 and R01 DK105954). H.X. was partially supported by a Training Grant in Alzheimer's Drug Discovery from the Lottie French Lewis Fund of the Community Foundation for Palm Beach and Martin Counties.

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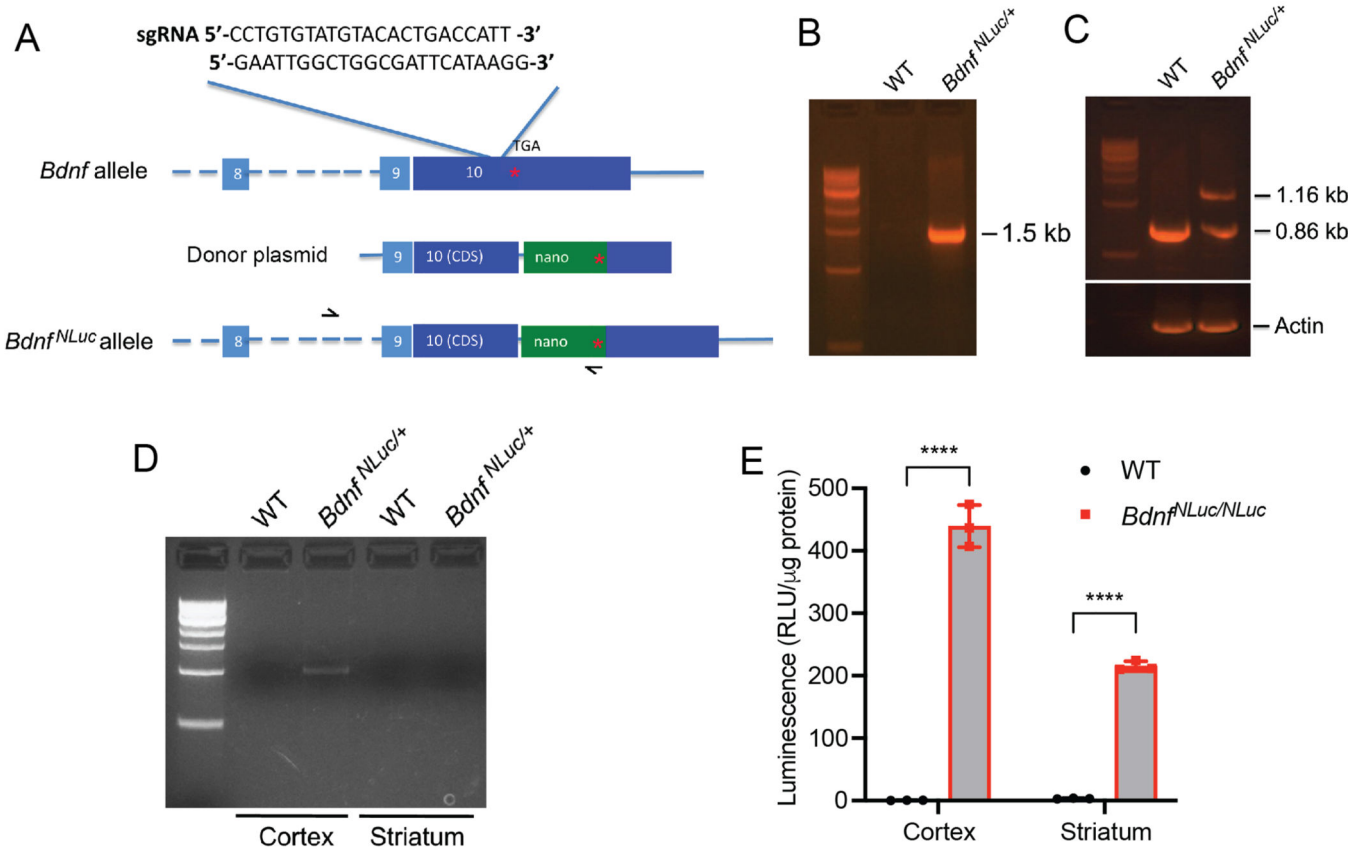

Figure 1.

Generation of *Bdnf*$^{NLuc/+}$ mice. (**A**) Two sgRNAs were used to increase the knocking-in efficiency. The donor plasmid was designed such that the NLuc coding sequence would be inserted immediately before the *Bdnf* stop codon. Red asterisks indicate the location of the stop codon. The two arrows denote the locations of two PCR primers used in (B). (**B**) Detection of the *Bdnf*^{NLuc} allele with genomic DNA PCR, using a forward primer outside the homology arm in the donor plasmid and a reverse primer complementary to the NLuc coding region. The PCR generates a 1.5-kb product from the *Bdnf*^{NLuc} allele. (**C**) RT-PCR detection of *Bdnf* mRNA variants in the cortex of WT and *Bdnf*$^{NLuc/+}$ mice. The forward primer is complementary to *Bdnf* exon 4, whereas the reverse primer is complementary to the *Bdnf* coding sequence for detecting exon 4 *Bdnf* mRNA variant (0.86 kb PCR product) or the NLuc coding sequence for detecting exon 4 *Bdnf-NLuc* mRNA variant (1.16 kb PCR product). (**D**) RT-PCR detection of *Bdnf* mRNA or *Bdnf-NLuc* mRNA in WT and *Bdnf*$^{NLuc/+}$ brain tissues. The forward primer is complementary to *Bdnf* exon 9, whereas the reverse primer is complementary to the NLuc coding sequence. (**E**) NLuc activities in cortical and striatal lysates. Unpaired t test, ****p<0.0001; n=3 female mice. Error bars represent SEM.

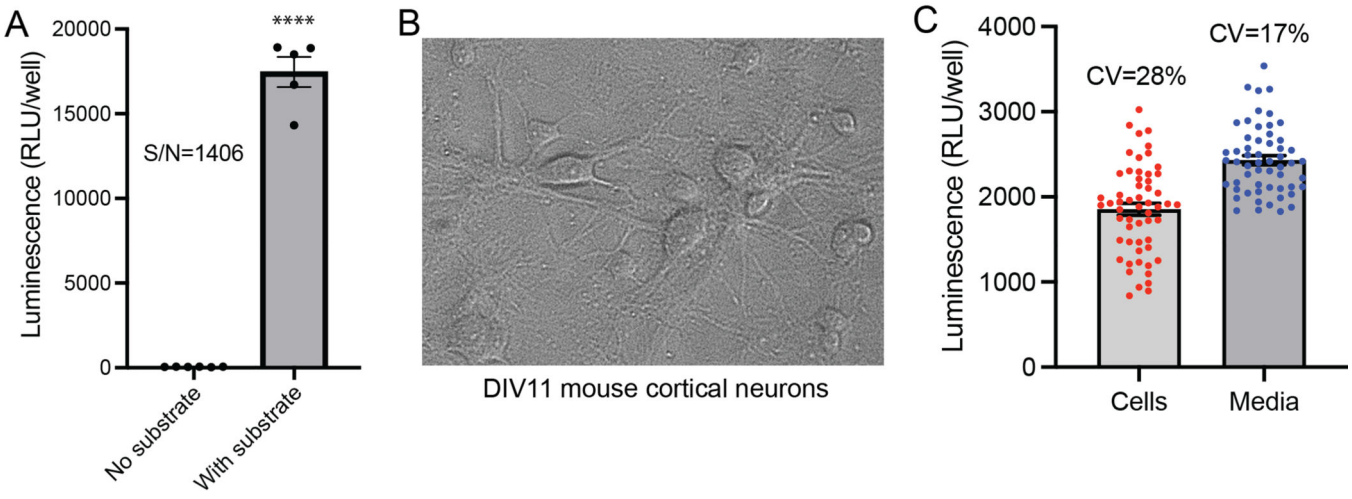

Figure 2.

Culturing cortical neurons from newborn *Bdnf*$^{NLuc/+}$ mice. (**A**) Activity of NLuc in DIV11 *Bdnf*$^{NLuc/+}$ neurons cultured in a 96-well plate. Unpaired t test, ****p<0.0001; n=5–6 wells. (**B**) Differential interference contrast image of DIV11 *Bdnf*$^{NLuc/+}$ neurons cultured in a 384-well plate. (**C**) NLuc activity in DIV11 *Bdnf*$^{NLuc/+}$ neurons cultured in a 384-well plate. NLuc activities in cells and media were measured separately (n=56 wells). Error bars represent SEM.

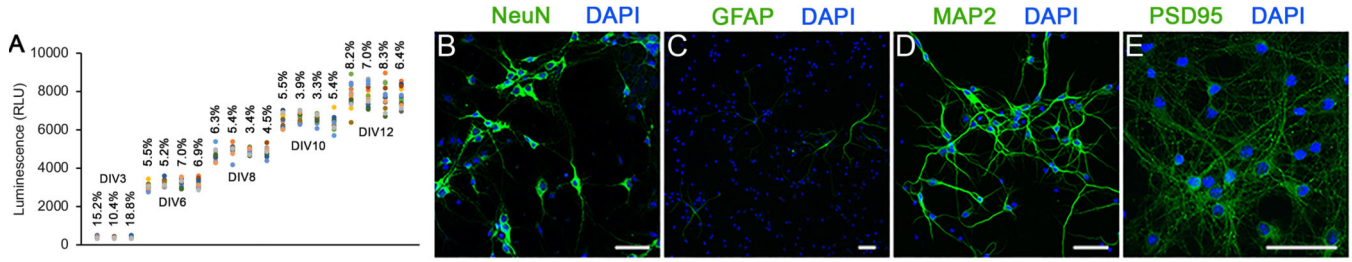

Figure 3.

Culturing embryonic *Bdnf*$^{NLuc/+}$ neurons in 384-well plates. (**A**) NLuc activity in media at different ages of neuronal cultures. Each well contained 80 µl of medium, 20 µl of which was used for measurement of NLuc activity. Each cluster of values represents NLuc activity from one row of wells except the two edge wells. CV for each row is shown above each cluster of value points. (**B-E**) Immunocytochemistry with antibodies against NeuN (neuronal marker), GFAP (astrocytic marker), MAP2 (dendritic marker) and PSD95 (postsynaptic marker) was performed on cultured cells at DIV7. Scale bars, 50 µm.

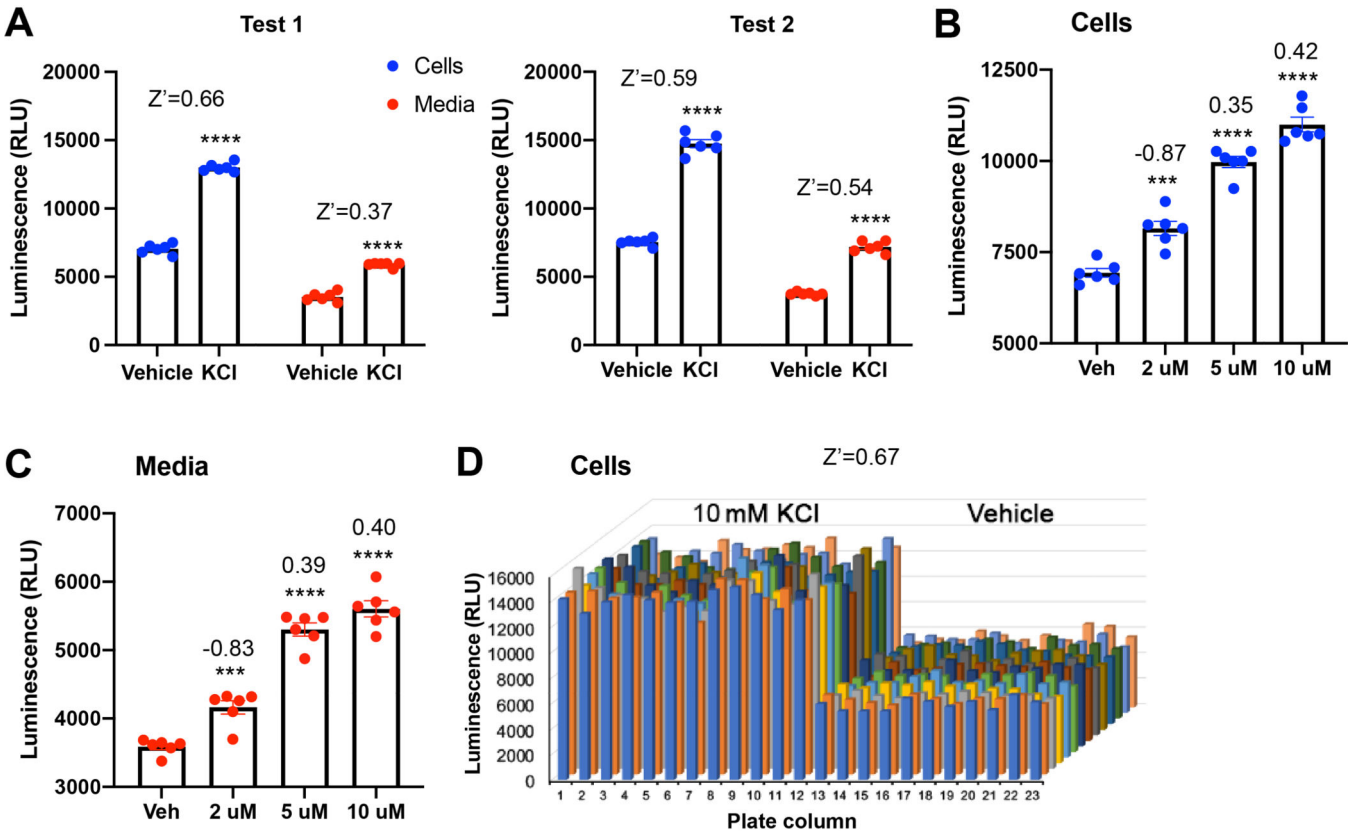

Figure 4.

KCl and DM stimulate BDNF synthesis in cultured *Bdnf*$^{NLuc/+}$ neurons. Chemicals were added on DIV7, and NLuc activities were measured on DIV8. (**A**) KCl at 10 mM increased levels of BDNF-NLuc in both neurons and media. The two tests were done in two different cultures. n=6 wells/condition. Z′ is calculated using the following formula: $1-(3\sigma_p + 3\sigma_n)/(|\mu_p - \mu_n|)$ where σ, μ, p and n stand for standard deviation, mean, positive control (KCl) and negative control (vehicle), respectively. Unpaired t test: **** p<0.0001. (**B, C**) DM at 2–10 μM increased levels of BDNF-NLuc in both neurons and media. The numbers above columns are Z factors. n=6 wells/condition. One-way ANOVA with Dunnett's multiple comparison test vs. vehicle: *** p<0.001 and **** p<0.0001. (**D**) DIV7 neuronal cultures in a 384-well plate were treated with either vehicle or 10 mM KCl, and NLuc activities were measured 24 hours later. Each bar represents the NLuc activity in cells of each well. Error bars represent SEM.

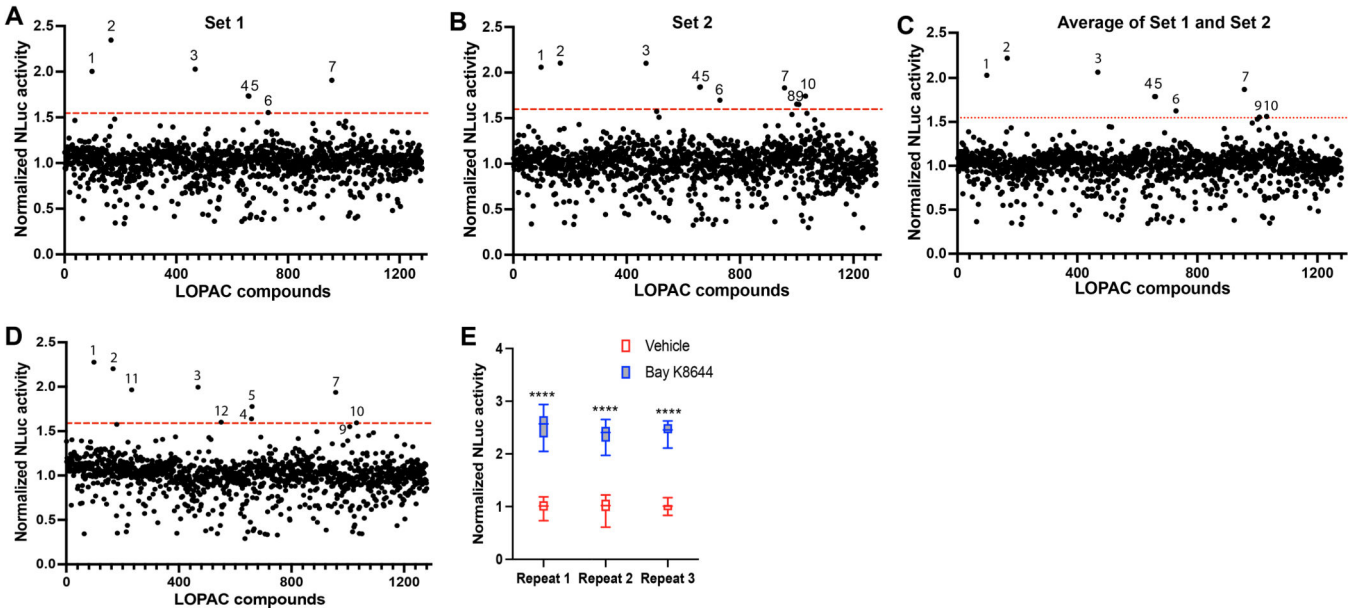

Figure 5.

Assay scale-up and assay repeatability. (**A**-**C**) LOPAC compounds were tested in 2 sets of 4 plates of cultured *Bdnf*$^{NLuc/+}$ neurons. The activity of NLuc in each well is normalized to the average of NLuc activities in 1280 compound-containing wells. Red dash lines indicate 3xSDs over the mean. Graphs show data from a single set of plates (A and B) or the average of the two sets of plates (C). (**D**) The graph shows results from an independent screening of LOPAC compounds with 4 plates of cultured *Bdnf*$^{NLuc/+}$ neurons. Red dash lines indicate 3xSDs over the mean. (**E**) Bay K8644 (10 μM) or vehicle (DMSO) was added to *Bdnf*$^{NLuc/+}$ neuronal cultures at DIV7 for 24 hours. Unpaired t test, ****p<0.0001, n=12 wells/condition. Identified compounds: 1, brefeldin A; 2, Bay K8644; 3, 1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole; 4, nitrendipine; 5, nimodipine; 6, N-propargyl nitrendipine; 7, GR127935 hydrochloride hydrate; 8, gabazine; 9, phorbol-12-myristate 13-acetate; 10, phenylbutazone; 11, PK11195; 12, felodipine.