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Loss of Huntingtin Function Complemented by Small Molecules Acting as Repressor Element 1/Neuron Restrictive Silencer Element Silencer Modulators^{*[S]}

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Dorotea Rigamonti^{†§1}, Daniele Bolognini^{†§1}, Cesare Mutti^{†1}, Chiara Zuccato[‡], Marzia Tartari^{‡2}, Francesco Sola^{¶1}, Marta Valenza^{‡2}, Aleksey G. Kazantsev^{||}, and Elena Cattaneo^{‡3}From the [†]Centre for Stem Cell Research and Department of Pharmacological Sciences, University of Milan, Via Balzaretti 9, Milan 20133, Italy, the [§]Dialectica S.r.l., c/o Nerviano Medical Sciences, V.le Pasteur 10, Nerviano MI 20014, Italy, the [¶]Biology Department, Nerviano Medical Sciences S.r.l., Building 75A, V.le Pasteur 10, Nerviano MI 20014, Italy, and the ^{||}MassGeneral Institute for Neurodegenerative Diseases, Massachusetts General Hospital, Charlestown, Massachusetts 02129-4404

Increased levels of the repressor element 1/neuron restrictive silencer element (RE1/NRSE) silencing activity promoter, and a consequent reduction in the transcription of many RE1/NRSE-bearing neuronal genes, including brain-derived neurotrophic factor (BDNF), have been demonstrated in Huntington disease (HD) and represent one possible effector of its selective neuronal vulnerability. Restoring the expression levels of neuronal genes in diseased neurons therefore seems to be an attractive therapeutic approach. To this end, we have developed a cell-based reporter assay for monitoring RE1/NRSE silencing activity and validated it by genetically inactivating the RE1/NRSE or pharmacologically stimulating global transcription. In a pilot compound screen, we identified three closely related structural analogues that up-regulate reporter expression at low nanomolar concentrations, and follow-up studies have shown that they efficaciously increase endogenous BDNF levels in HD cells. Moreover, one of the compounds increases the viability of HD cells. Our findings suggest a new avenue for the development of drugs for HD and other neurodegenerative disorders based on the pharmacological up-regulation of the production of the neuronal survival factor BDNF and of other RE1/NRSE-regulated neuronal genes.

Huntington disease (HD)⁴ is an inherited dominant neurodegenerative disorder for which there is no effective treatment. Since the discovery that its genetic cause is a polyglutamine expansion in the novel protein huntingtin (1), extensive efforts have been made to find its underlying mechanisms and potential therapeutic targets.

A number of pathogenic mechanisms have been considered so far, which indicates that more than one target should be investigated to reach a positive therapeutic effect (2–4). Most of these targets have been identified by looking at disease mechanisms triggered by *gain of function* defects caused by mutant huntingtin (5), and we and other groups have hypothesized that diminished wild-type huntingtin function may also contribute to the pathogenesis of HD (6). This suggests that a complementary approach based on identifying compounds that mimic wild-type huntingtin activity should be considered: the administration of novel compounds capable of restoring wild-type huntingtin function, together with drugs reducing mutant huntingtin toxicity, may be an optimal therapeutic strategy.

The repressor element 1/neuron restrictive silencer element (RE1/NRSE) was the first example of an affected target of wild-type huntingtin activity to be discovered and is a silencer of gene expression (7–9). Bioinformatic studies indicate that more than 1000 neuronal genes in the human genome bear an RE1/NRSE sequence in their promoters (10, 11), of which brain-derived neurotrophic factor (BDNF) is particularly interesting, because it is a pro-survival factor for the striatal neurons that die in HD. We have previously shown that wild-type huntingtin stimulates BDNF gene transcription (12) by retaining in the cytoplasm the transcription factor that binds and activates the silencing transcription factor/neuron-restrictive silencer factor (REST/NRSF) sites involved in the regulation of RE1/NRSE and located inside BDNF promoter II (13). The inactivation of RE1/NRSE sites allows BDNF gene transcription (13, 14). Mutant

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^[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. S1.

¹ Present address: Dialectica S.r.l., c/o Nerviano Medical Sciences, V.le Pasteur 10, Nerviano MI 20014, Italy.

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³ To whom correspondence should be addressed. Tel.: 39-02-5031-8333; Fax: 39-02-5031-8284; E-mail: elena.cattaneo@unimi.it.

⁴ The abbreviations used are: HD, Huntington disease; RE1/NRSE, repressor element 1/neuron restrictive silencer element; BDNF, brain-derived neurotrophic factor; REST/NRSF, silencing transcription factor/neuron-restrictive silencer factor; HDAC, histone deacetylase; CRF, corticotropin-releasing factor; RLU, relative light unit; ELISA, enzyme-linked immunosorbent assay; DAPI, 4',6'-diamidino-2-phenylindole; BrdUrd, bromodeoxyuridine; ANOVA, analysis of variance; DN, dominant negative; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TSA, trichostatin A; SAHA, suberoylanilide hydroxamic acid.

huntingtin is less capable of recruiting REST/NRSF in the cytoplasm, which leads to its pathological translocation to the nucleus and increased occupancy of its cognate RE1/NRSE genomic loci (13, 14). In HD, the occupied RE1/NRSE silences transcription from RE1/NRSE-regulated genes, thus contributing to neuronal dysfunction (13, 14).

Interest in compounds acting on a transcriptional system that supports the transcription of the BDNF gene (among others) is sustained by the fact that positive outcomes have been observed in HD models after increasing BDNF levels by means of transplanting cells engineered to overexpress BDNF into a chemical model of HD (15) or delivering BDNF via gene therapy (16, 17). Transgenic overexpression of BDNF in the cerebral cortex can delay the progression of disease phenotypes in the R6/1 HD mouse model carrying the first 63 amino acids of mutant human huntingtin protein (18), and the genetic reduction of BDNF in the same R6/1 mice causes a worse phenotype (19). Furthermore, these findings are paralleled by the fact that mice lacking cortical BDNF show a hind limb clasping phenotype similar to that observed in mouse models of HD (20). Finally, it has been more recently demonstrated that cystamine improves the HD phenotype in mice by enhancing BDNF release from the Golgi compartment in brain cells (21).

We here describe the development of the first cell-based assay designed to screen for compounds that are potentially capable of restoring the loss of wild-type huntingtin function in RE1/NRSE-mediated gene transcription by identifying genetic and chemical RE1/NRSE modulators. We used this assay to identify some small molecules that inhibit RE1/NRSE activity and then assessed their efficacy on molecular and phenotype parameters in HD cellular models.

EXPERIMENTAL PROCEDURES

Transcription Factor and Reporter Gene Constructs—The RE1/NRSE^{bdnf}-LUC reporter construct was constructed using the enhanced luciferase reporter gene pGL3-promoter (Promega, Madison, WI) as a backbone. A 300-bp portion of the rat BDNF promoter exon II containing the RE1/NRSE sequence was obtained from the BDNF+II CAT construct (41), and subcloned into the Xho-HindIII digested pGL3-promoter from which SV40 was excised. The same cloning strategy was used for the mutated RE1/NRSE^{bdnf}-LUC. The 300-bp insert was obtained from the BDNFI+II^{mut} CAT construct (42). The mutated nucleotides in the mutated RE1/NRSE are described in Timmusk *et al.* (42). The DN:REST and REST/NRSF (22, 10) expression constructs were kindly provided by Noel J. Buckley, University of Leeds, UK.

Cell Cultures, Engineering, and Antibiotic Selection—ST14A and ST^{HdhQ111/HdhQ111} cells were grown in high glucose Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum at 33 °C in a 5% CO₂ atmosphere (43). For the transient transfection experiments, the harvested ST14A cells were resuspended in electroporation medium (Dulbecco's modified Eagle's medium) at a density of 625,000 cells/ml, and 400 μ l of the cell suspension was put into an electroporation cuvette together with plasmids (see "Results" for the amounts). The cells were electroporated using a BTX ElectroSquare PoratorTM ECM 830 at 160 V for 30 ms and

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one pulse, after which they were diluted in complete medium and plated in 24-well plates.

To obtain stable clones, 8 μ g of the RE1/NRSE^{bdnf}-LUC reporter construct and 2 μ g of pLXSP for puromycin resistance were delivered to one million neural cells. After electroporation, the cells were plated in 100-mm Petri dishes and a selection medium containing 3 μ l/ml puromycin was applied 24 h later. After 15 days of selection pressure, 25 single clones were transferred to 24-well plates and expanded. The subclones were tested for basal luciferase activity and, in a second step, those that were positive were electroporated with DN:REST (5 μ g) to detect subclones expressing a functional and responsive RE1/NRSE^{bdnf}. For DN:REST infection, 109 plaque-forming units/ml of virus particles were used to infect 145,000 cells.

Chemicals and Chemical Exposure—A panel of compounds of interest to test in the developed assay was assembled. Small molecules known to be pan-HDAC inhibitors (TSA or SAHA) and other transcriptional activators were included, in addition to entities that might modulate BDNF pathway and expression. CRF, CRF antagonist, forskolin, azacitidine-A, TSA, SAHA, and tranlylcipromine were purchased from Sigma. Compounds 2, 18, and 19 and amonafide and its derivatives were purchased from Chembridge Corp. (San Diego, CA). The compounds were dissolved in Me₂SO and kept in 10 mM stock solutions before being added at the indicated doses to the cells plated in on the day before. The mRNA or proteins were collected for the different experiments at the indicated time points.

Determination of Luciferase Expression—In transient experiments, *Renilla* was used to normalize the transfection efficiency data, and a Dual Luciferase Reporter Assay System was used according to the manufacturer's protocol (Promega). *Renilla* and firefly luciferase activities were determined using the Veritas Microplate Luminometer (Promega), counting each sample for 8 s. The results are given as firefly/*Renilla* relative light units (RLUs).

Alternatively, after the addition of luciferin as a substrate (Beetle Luciferin, Promega), firefly luciferase activity was determined using the luminometer and is given as RLUs in relation to protein content, as assessed using a bicinchoninic acid assay (Pierce).

Gene Expression Analyses—Three independent room temperature reactions were set up for each RNA stock of 1 μ g of total RNA preparations, and gene expression was assessed by means of real-time PCR. The analyses were made in triplicate for each of the genes to obtain replicates for statistical analysis.

We used an iCycler Thermal Cycler with a multicolor real-time PCR detection system (Bio-Rad). All of the reactions were performed in a total volume of 25 μ l, containing 25 ng of cDNA, 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 0.2 mM dNTPs, 25 units/ml iTaq DNA polymerase, 3 mM MgCl₂, 10 nM SYBR Green I fluorescein, stabilizers (iQTM SYBR Green Supermix-Bio-Rad), and 0.2 μ M of forward and reverse primers.

The amplification cycles for total BDNF consisted of an initial 10-min denaturing cycle at 95 °C, followed by 40 cycles of 10 s at 95 °C, 10 s at 56 °C, and 20 s at 72 °C. Fluorescence was quantified during the 56 °C annealing step, and product formation was confirmed by means of a melting curve analysis (55–94 °C). The amounts of target gene mRNA were normalized to

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a reference gene (β -actin) amplified following the same protocol. The primer sequences were: BDNF-F, 5'-GAT GAG GAC CAG AAG GTT CG-3'; BDNF-R, 5'-GAT TGG GTA GTT CGG CAT TG-3'; β -actin-F, 5'-AGT GTG ACG TTG ACA TCC GTA-3'; and β -actin-R, 5'-GCC AGA GCA GTA ATC TCC TTC T-3' (F, forward; R, reverse).

The amplification cycles for synapsin I and M4 muscarinic receptor consisted of an initial 3-min denaturing cycle at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C. Fluorescence was quantified during the 60 °C annealing step, and product formation was confirmed by means of a melting curve analysis (55–94 °C). The primer sequences were: synapsin I-F, 5'-TTT TTG GGG GAC TTG ACA TC-3'; synapsin I-R, 5'-AGT TCC ACG ATG AGC TGC TT-3'; M4 muscarinic-F, 5'-GGA ACC TCT GGC TTG TTC C-3'; M4 muscarinic-R, 5'-CAG ACT GAT TGG CTG AGC TG-3'.

The amplification cycles for E2F1 consisted of an initial 10-min denaturing cycle at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Fluorescence was quantified during the 60 °C annealing step, and product formation was confirmed by means of a melting curve analysis (55–94 °C). The primer sequences were: E2F1-F, 5'-CCC GCC GGT GAA ACG-3'; E2F1-R, 5'-GCC TCT GCC CCG GAA T-3'.

The amplification cycles for REST/NRSF consisted of an initial 3-min denaturing cycle at 95 °C, followed by 45 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Fluorescence was quantified during the 60 °C annealing step, and product formation confirmed by a melting curve analysis (55–94 °C). The primer sequences were: REST/NRSF-F, 5'-CGA ACT CAC ACA GGA GAA CG-3'; REST/NRSF-R, 5'-GAG GCC ACA TAA TTG CAC TG-3'. All the amounts of target gene mRNA were normalized to a reference gene (β -actin) according to a previous study (44).

ELISA Assay—Cell lysates were prepared in lysis buffer consisting of glycerol 10%, 25 mM Tris HCl, pH 7.5, 150 mM NaCl, Triton X-100 1%, 5 mM EDTA, 1 mM EGTA supplemented with 1:100 of Protease Inhibitor Mixture (Sigma). The samples were homogenized, sonicated, and centrifuged (Biofuge, 15 min at 4 °C at maximum speed), and the supernatants were collected and stored at –80 °C. The samples were assayed for BDNF using an ImmunoAssay System (Promega) as described by the manufacturer. The proteins were quantified by means of a bicinchoninic acid protein assay (Pierce) before loading.

Cell Viability Assay—80,000 cells per well were plated in triplicate into 24-well plates. After 24-h incubation at 33 °C in 5% CO₂, the cultures were washed with Hanks' balanced salt solution, and the medium was replaced by serum-deprived medium and then incubated at 37 °C. At the indicated time points, the cells were exposed to 3-(4,5-dimethylthiazol-2-phenyl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich), and the mitochondrial release of formazan was quantified at 560 nm after incubation at 37 °C for 1 h.

Immunocytochemistry—4500 ST^{HdhQ111/HdhQ111} cells were plated in 96-well Cellomics plates, coated with poly-lysine, and, after adhesion, exposed to the selected compound. At the indicated time points, the cells were fixed in paraformaldehyde 4% for 15 min, washed with 1× phosphate-buffered saline and then permeabilized with 0.5% Triton X-100 in 1× phosphate-buff-

ered saline. They were blocked for 1 h at room temperature in 1× phosphate-buffered saline, 5% nonfat milk, and 5% goat serum. The cells were exposed to primary antibody (1:500) rabbit polyclonal anti-REST (Upstate) for 1 h at room temperature, followed by the goat anti-rabbit FluoroLink Cy2 (Amersham Biosciences) secondary antibody (1:500) for 2 h. The nuclei were stained blue with DAPI. Automatic image capture and analysis were performed using a Cellomics Array Scan IV (Thermo Fisher) with the Cytoplasm to Nucleus Translocation BioApplication. For the BrdUrd incorporation study, ST^{HdhQ111/HdhQ111} cells were exposed to stress stimuli in the presence or absence of compound 18. BrdUrd incorporation was performed over the last 24 h, and the cells were fixed with paraformaldehyde 4% for 15 min and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline for 5 min at room temperature. After DNA denaturation, the cells were stained using anti-BrdUrd monoclonal antibody (BD Biosciences). For DNA staining, fixed cells were incubated with DAPI. A total of 200 cells was counted for each sample.

Statistical Analysis—Bonferroni's test or an ANOVA *t* test were used as indicated to evaluate the statistical significance of the results.

RESULTS

Development of the RE1/NRSE^{bdnf} Luciferase Reporter Assay—We initially evaluated the functioning of the RE1/NRSE^{bdnf} luciferase construct and its responsiveness to REST/NRSF.

ST14A neural cells, which are known to express endogenous REST/NRSF (13), were transiently transfected with the reporter construct and a dominant negative REST/NRSF construct (DN:REST) (22). The latter consists of the DNA binding domain of REST/NRSF, but lacks the repressor domain, and was expected to attenuate REST/NRSF binding to RE1/NRSE^{bdnf} within the luciferase reporter and increase luciferase activity. Forty-eight hours after transfection, there was a proportional increase in luciferase activity with increasing doses of DN:REST in comparison with untransfected cells or cells treated with an empty vector (Fig. 1A), thus indicating that DN:REST overexpression attenuates endogenous REST/NRSF binding to RE1/NRSE^{bdnf} in a dose-dependent manner. When co-transfected with RE1/NRSE^{bdnf} luciferase, REST/NRSF counteracted the luciferase expression induced by DN:REST (Fig. 1A, *light gray column*).

Consistently, when present in the promoter controlling the reporter (mRE1/NRSE^{bdnf}-LUC), a specific mutation in the RE1/NRSE sequence abolished the effect of DN:REST (Fig. 1B). It is also worth noting that mRE1/NRSE^{bdnf}-LUC transfection gave rise to a stronger luciferase signal than RE1/NRSE^{bdnf} because of the lack of the silencing activity of mutant RE1/NRSE^{bdnf} (Fig. 1B, *third versus first column*). We therefore concluded that the RE1/NRSE^{bdnf} luciferase reporter is functional and specifically responsive to REST/NRSF transcription factor.

Generation and Genetic Validation of the Stable DiaNRSE^{Luc8} Reporter Cell Line—To obtain stable cell subclones, the ST14A neural cell line was co-transfected with RE1/NRSE^{bdnf} luciferase and pLXSP vector. After antibiotic selection, 25 puromycin-resistant subclones were isolated, and the basal expression of

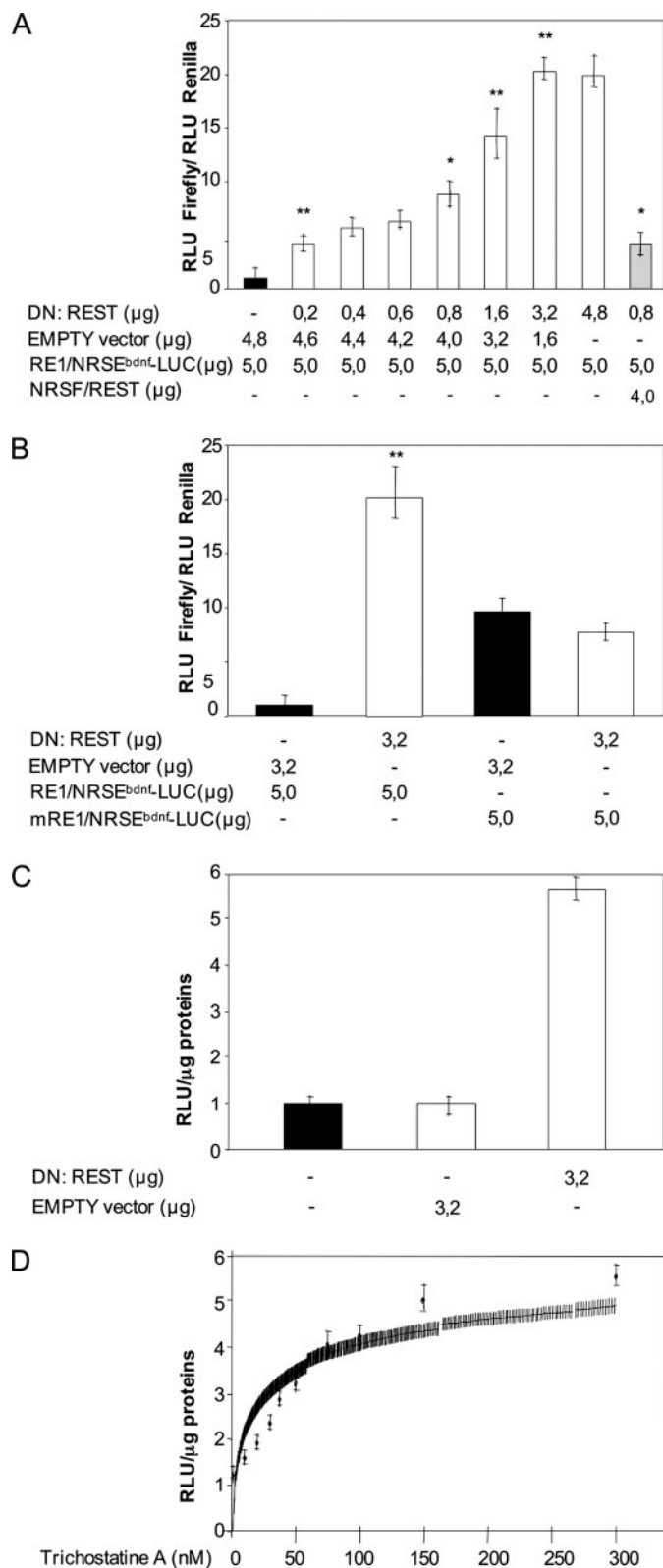


FIGURE 1. RE1/NRSE^{bdnf} within a luciferase construct is responsive to REST/NRSF. A, ST14A cells were transiently co-transfected with the RE1/NRSE^{bdnf} luciferase construct and the indicated amount of DN:REST; after 48 h, there was a dose-dependent increase in luciferase activity (Bonferroni's test). The presence of exogenous REST/NRSF competes with DN:REST, thus decreasing reporter induction (light gray column). Experiments were performed in triplicate; the data are shown as firefly/Renilla RLUs in relation to controls (black column). *, $p < 0.05$; **, $p < 0.01$ versus the immediately lower dose. B, ST14A cells were transiently co-transfected with the RE1/NRSE^{bdnf}

the transgene was evaluated by means of a luciferase assay; 13 subclones were discarded because of the decrease in luciferase activity over multiple passages.

The remaining subclones were transiently transfected with DN:REST or an empty vector, and their luciferase activity was tested to select subclones stably expressing a functional and responsive RE1/NRSE^{bdnf} luciferase reporter. 8 of the 12 clones showed no more than a 1.5-fold increase in luciferase expression, which we thought might be due to effects of the surrounding chromatin at the transgene integration sites. However, DN:REST significantly increased luciferase activity in the remaining four clones, and we selected the one with the best signal-to-background ratio for further characterization. Fig. 1C shows that this clone (designated DiaNRSE^{Luc8}) specifically responds to DN:REST infection with a 6-fold increase in luciferase activity.

Pharmacological Evaluation of DiaNRSE^{Luc8} Cell-based Assay—As REST/NRSF recruits HDACs and acts as a repressor through chromatin remodeling (23, 24), the assay response was tested in the presence of the known transcriptional activators: pan-HDAC inhibitors TSA and SAHA.

In particular, when DiaNRSE^{Luc8} cells were exposed to TSA at concentrations of up to 300 nM, there was a dose-dependent increase in luciferase activity (Fig. 1D). The effect of TSA indicates that HDAC activity is required for the repression of RE1/NRSE^{bdnf} by REST/NRSF, and that the RE1/NRSE^{bdnf} within the luciferase reporter is responsive to an HDAC inhibitor. For this reason, we selected TSA as the reference compound for further assay development.

After scaling down the assay to 96 wells, we calculated the Z' factor, a dimensionless measure of the quality of a high-throughput screening assay that considers the mean signal and standard deviation from positive and negative controls (25). The calculated Z' factor value from 20 independent experiments in which DiaNRSE^{Luc8} cells were exposed to 75 nM of TSA for 72 h was 0.81 ± 0.12 , thus indicating the reliability of the assay.

The basis for the transcriptional response of the developed assay was evaluated in a pilot drug screening study of a panel of selected compounds. In the search for a de-repressor of transcription, we first used compounds that are known to possess this activity. We included a DNA-demethylating agent, azacitidine-A, which activates gene transcription by reducing the stability of silencing signals, and amonafide and its structurally

luciferase construct bearing a mutated RE1/NRSE (mRE1/NRSE^{bdnf}-LUC) and DN:REST; after 48 h, there was no increase in luciferase activity, thus confirming that the induction observed in the presence of RE1/NRSE^{bdnf}-LUC is specifically due to RE1/NRSE^{bdnf}. The data are shown as firefly/Renilla RLUs in relation to controls (RE1/NRSE^{bdnf}-LUC transfected cells, first black column). The experiments were performed in triplicate; **, $p < 0.01$, Bonferroni's test. Genetic and chemical validation of the DiaNRSE^{Luc8} clonal cell line. C, when the DiaNRSE^{Luc8} subclone was infected with DN:REST adenoviral supernatant, there was a 5.5-fold increase in luciferase activity 48 h after infection. The data are shown as firefly RLUs/ μ g of protein in relation to controls (uninfected cells, black column). D, when the DiaNRSE^{Luc8} subclone was exposed to increasing amounts of TSA (an HDAC inhibitor that is effective at nanomolar concentrations *in vitro*), there was a dose-dependent increase in luciferase activity; the concentration of 75 nM was selected for further assay development. The data are shown as firefly RLUs/ μ g of protein in relation to controls (untreated cells) and are the average of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ versus the immediately lower dose, Bonferroni's test.

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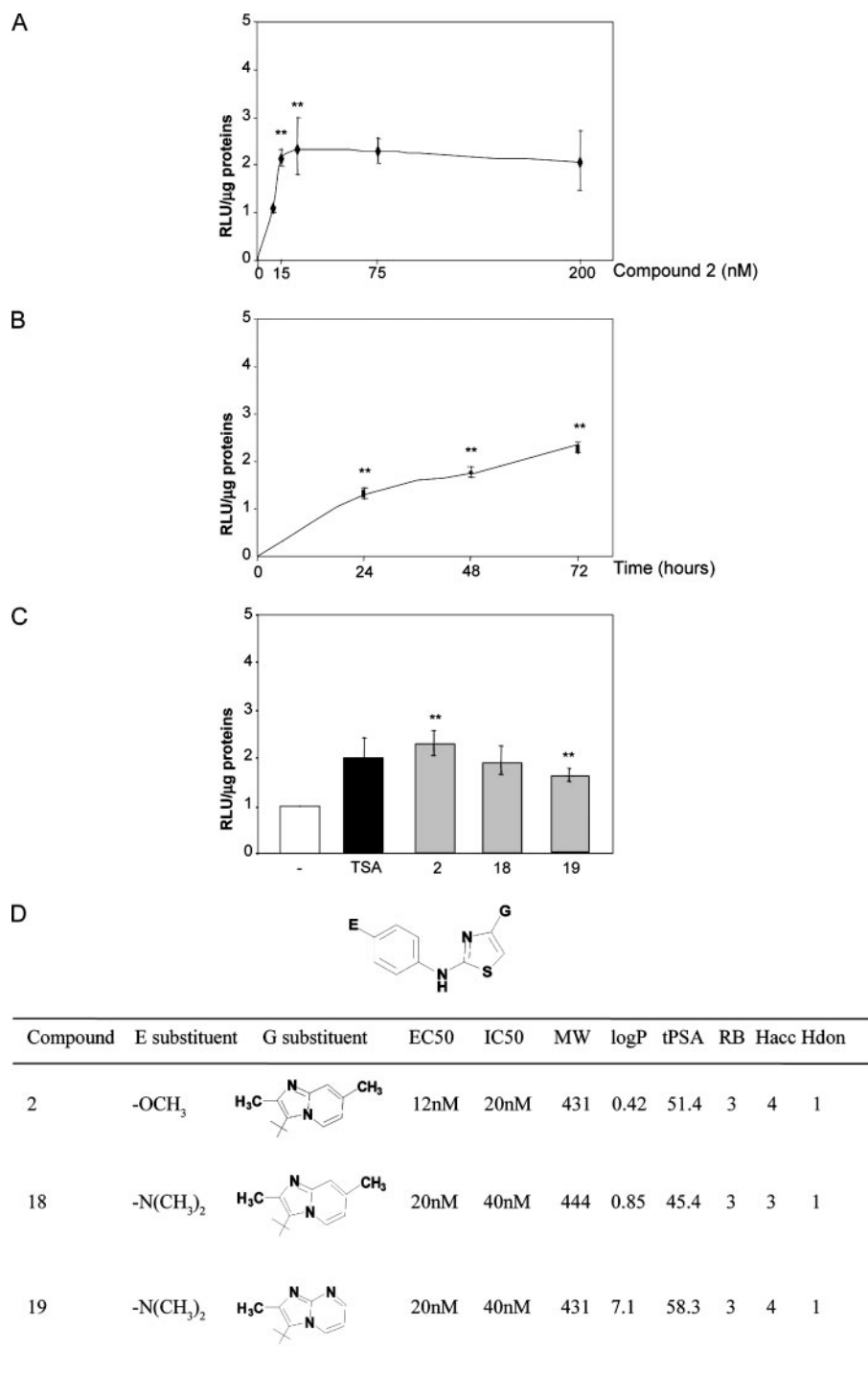


FIGURE 2. Pilot drug screening assay. Luciferase activity in the DiaNRSE^{Luc8} subclone significantly increased after the administration of the selected compounds. *A*, representative dose response: **, $p < 0.01$ versus the lower dose, Bonferroni's test; *B*, time course: **, $p < 0.01$ versus time 0, ANOVA. The curves are those of compound 2. *C*, luciferase activity in DiaNRSE^{Luc8} after the administration of 75 nM of compounds 2, 18, or 19. The data are the average of three independent experiments: **, $p < 0.01$ versus untreated cells, ANOVA. *D*, EC₅₀ assessed by reporter activity, and IC₅₀ assessed by MTT assay, describing cell viability 72 h after exposure to the indicated compounds. Physical/chemical data are shown for the indicated structures.

similar derivatives of the C91 series, which have previously been identified as increasing transcriptional activity in a reporter assay of mutant huntingtin knock-in cells in which the reporter was regulated by six Specificity protein-1 binding sites (26). Small molecules targeting the cell pathways involved in regulating BDNF expression were also chosen, in particular for-

skolin, an activator of adenylate cyclase, because an increase in cAMP levels enhances BDNF expression (27). As it is known that antidepressants also increase BDNF expression levels (28, 29), we selected tranylcipromine, a monoamine oxidase inhibitor; and as some published findings indicate that CRF stimulates BDNF expression (30, 31), we included a structural analogue of known inhibitors of CRF receptor (32, 33).

DiaNRSE^{Luc8} cells were plated in 96-well dishes and, after adhesion, exposed to the chemical entities in triplicate for 72 h, after which luciferase activity was evaluated. The compounds were initially tested at 0.1, 1, 5, and 10 μM . None of the compounds positively modulated reporter activity in our assay, except for the structural analogue of CRF receptor inhibitors (referred to as compound 2 in this report), which proved to be highly potent although toxic in the assay. Compound 2 was further tested in a dose-dependent experiment at nanomolar concentrations, which revealed a corresponding increase in luciferase activity from the lowest to the highest dose (Fig. 2A). Fig. 2B shows the increase in luciferase activity induced by 25 nM of compound 2 over time. Given the specificity of the result, we next tested a peptide-based CRF receptor agonist and antagonist, which had no effects on luciferase expression (RLU/ μg : untreated control 1: CRF, 200 nM 0.91 ± 0.02 and 1 μM 0.88 ± 0.02 ; CRF-antagonist, 200 nM 0.81 ± 0.01 and 1 μM 0.83 ± 0.01) and thus, in our view, ruled out the CRF-dependent mechanism of activation. This conclusion is supported by recently published data concerning the absence of any effects of CRF antagonists on BDNF production in rats (34).

To ensure the specificity of compound 2, which was identified empirically as being capable of stimulating luciferase expression, we tested some structurally similar and commercially available analogues. Compounds 18 and 19, whose ability to induce luciferase activity paralleled the effect observed in the presence of the positive control, TSA 75 nM (Fig. 2C, black column) as observed for compound 2, were

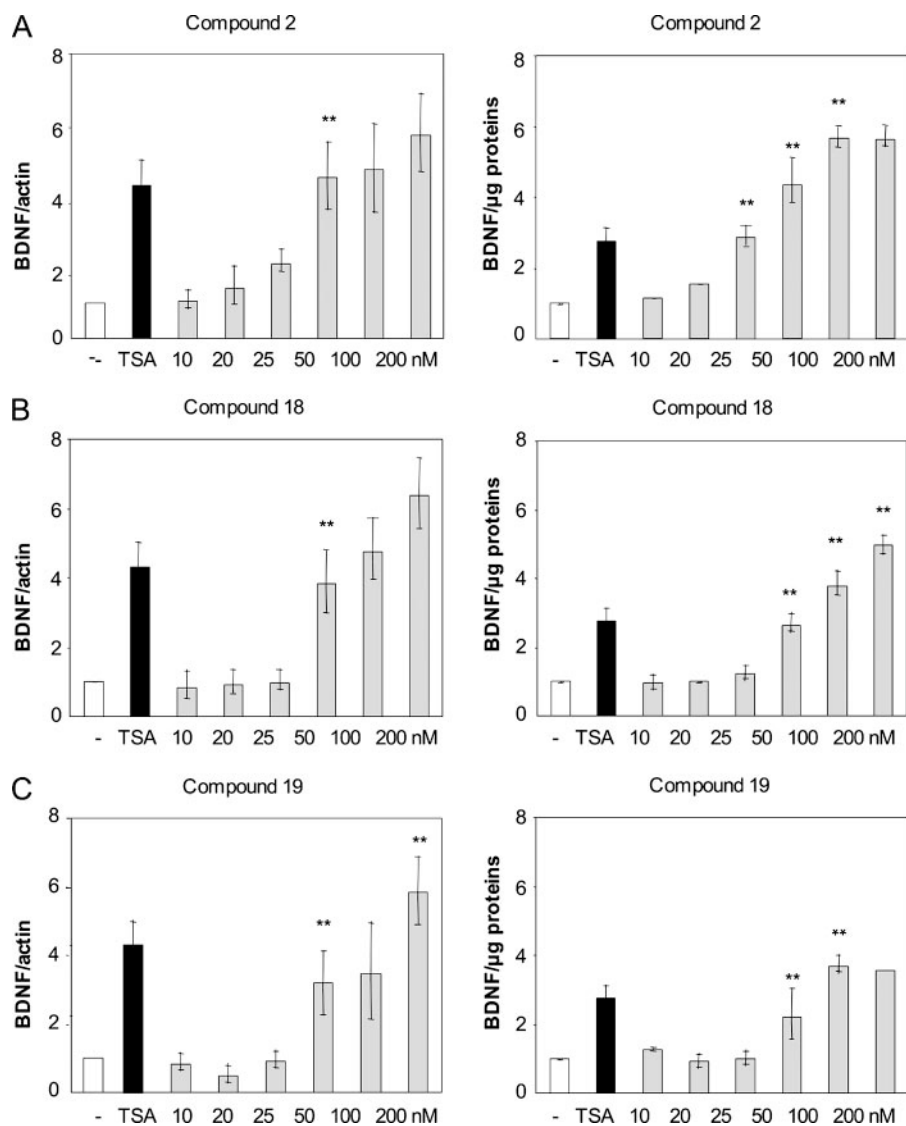


FIGURE 3. Secondary assay: compound effects on endogenous BDNF mRNA and protein levels. ST14A cells were exposed to the indicated amount of the three compounds, and, after 72 h, BDNF mRNA and protein levels were evaluated by means of quantitative real-time PCR and ELISA. The *left panels* show the quantitative expression of BDNF mRNA in comparison with β -actin (-fold increase in relation to untreated cells); the *right panels* show the quantitative evaluations of BDNF protein levels in relation to total protein content (-fold increase in relation to untreated cells). The data are the average of three independent experiments; **, $p < 0.01$ versus the immediately lower dose, Bonferroni's test. *A*, BDNF mRNA (*left*) and protein levels (*right*) after the administration of compound 2; *B*, BDNF mRNA (*left*) and protein levels (*right*) after the administration of compound 18; *C*, BDNF mRNA (*left*) and protein levels (*right*) after the administration of compound 19.

then tested in time-course and dose-response experiments and showed similar profiles to those observed with compound 2 (data not shown). Fig. 2*D* shows the structures and properties of the three compounds, together with the data relating to their activity (EC_{50} , luciferase activity) and toxicity (IC_{50} , cell viability as assessed by MTT assay). To ensure that the apparent cytotoxicity of the compounds was not interfering with, or responsible for, transcriptional activation, DiaNRSE^{Luc8} cells were exposed to 3-nitropropionic acid (5 and 10 nM) or staurosporine (10 nM), which are known to induce cell death in this neural cell line (35). As shown, neither increased luciferase activity (supplemental Fig. S1). Further interest in investigating the novel structural scaffold was aroused by the *in silico* prediction that compounds 18 and 19 cross the blood-brain barrier.

Secondary Assay—To evaluate whether the reporter assay can identify compounds that inhibit the activity of the endogenous RE1/NRSE loci, mRNA was collected from ST14A neural cells exposed to compounds 2, 18, or 19. Real-time PCR showed an increase in total BDNF mRNA after the administration of all three compounds (Fig. 3, *left*), and an ELISA assay in the same experimental paradigm showed that the rescue of BDNF mRNA was mirrored by an increase in BDNF protein levels. The *right panel* of Fig. 3 shows the intracellular BDNF protein content measured in cell lysates collected under basal conditions (*white column*), after 72-h exposure to TSA as positive control (*black column*), and in the presence of the indicated amount of the different compounds. Bonferroni's test showed that the increase in endogenous BDNF transcription and translation is dose-dependent ($p < 0.01$).

Compounds 2, 18, and 19 Increase Synapsin and M4 Muscarinic Receptor Gene Expression but Do Not Affect REST/NRSF or E2F1 Gene Transcription—To demonstrate that the compounds selected using DiaNRSE^{Luc8} cells can induce the RE1/NRSE-regulated expression of genes other than BDNF, synapsin I and M4 muscarinic receptor expression were evaluated as two of the known neuronal genes comprising a RE1/NRSE.

The mRNA collected from parental ST14A cells (exposed to the different compounds using the same experimental conditions as above) was retrotranscribed, and the cDNA was amplified using specific primers for the two genes. The results (Fig. 4, *A* and *B*) showed that exposure to 200 nM of compounds 2, 18, or 19 led to a statistically significant increase in synapsin I mRNA expression, and an increase was also observed in the case of the M4 muscarinic receptor.

To exclude the possibility that the compounds act as broad transcriptional activators, we analyzed their effects on the expression of two genes that are not listed among the RE1/NRSE-regulated genes: REST/NRSF and E2F1. Real-time PCR (Fig. 4, *C* and *D*) revealed a substantial increase in the cells treated with the SAHA HDAC inhibitor, whereas the REST/NRSF and E2F1 genes were not modulated by compounds at doses that are active on RE1/NRSE genes, thus suggesting that the compounds act selectively on RE1/NRSE-regulated gene transcription.

Identification of RE1/NRSE Silencer Modulators

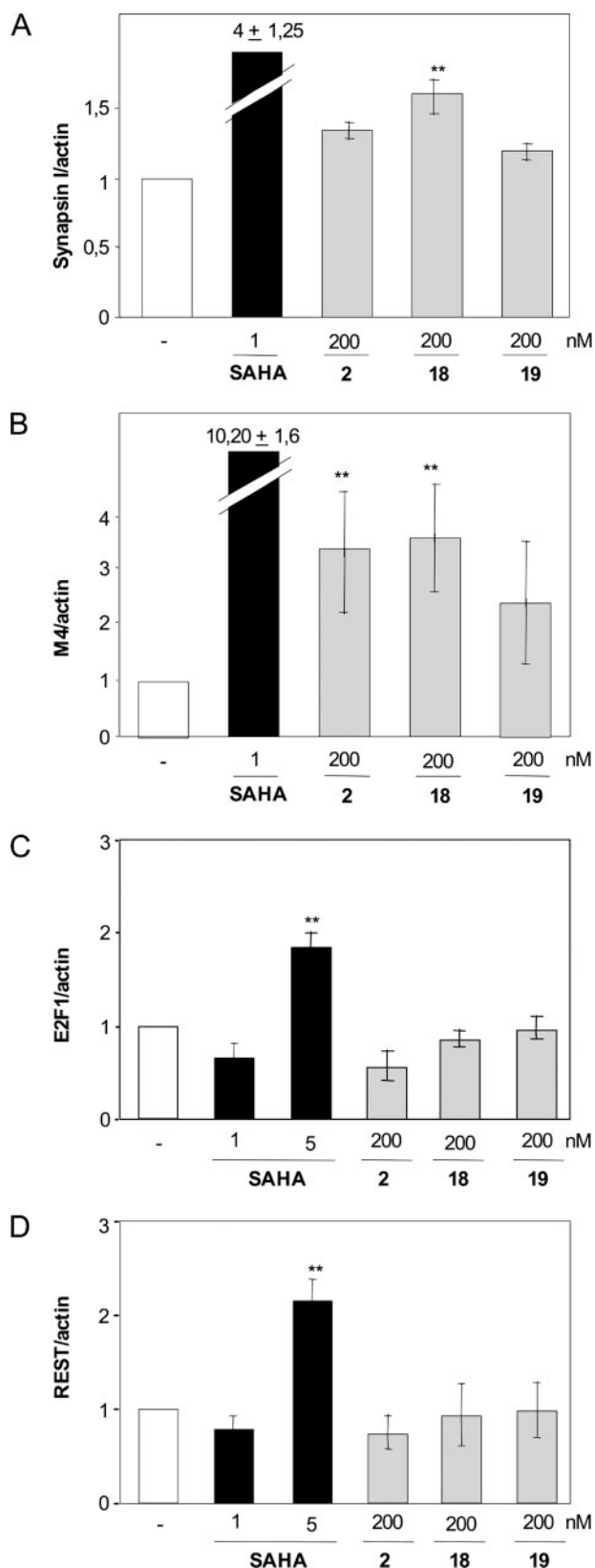


FIGURE 4. The compounds affect synapsin I and M4 muscarinic RE1/NRSE-regulated genes but not non-RE1/NRSE-regulated genes such as REST/NRSF and E2F1. ST14A cells were exposed to the indicated concentrations of the three selected compounds for 72 h, and the mRNA levels of synapsin I (A) and M4 muscarinic receptor (B) (two known RE1/NRSE-regulated genes), and E2F1 (C) and NRSF/REST (D) (two non-RE1/NRSE-regulated genes) were evaluated by means of quantitative real-time PCR. The graphs show quantitative

Compounds 2, 18, and 19 Restore BDNF mRNA and Protein Levels in an HD Context—We next tested whether the compounds increased BDNF gene transcription in the presence of mutant huntingtin using two previously derived neural cell lines that showed a decrease in BDNF content in comparison with their control counterparts (12).

In a first step, compounds 2, 18, or 19 were administered to neural cell lines derived from homozygous mutant huntingtin knock-in mice in which an expanded CAG has been inserted into the endogenous mouse *Hdh* gene ($ST^{HdhQ111/HdhQ111}$) (36). Fig. 5A shows the ELISA results indicating an ~2-fold increase after treatment with 200 nM of compounds 2 and 18, whereas 200 nM of compound 19 did not further increase the 1.6-fold increase observed after treating the cells with 50 nM of the same compound. The selected compounds also increased BDNF protein content in independent neural cell lines engineered in such a way as to stably overexpress the full-length mutant huntingtin protein (35) (Fig. 5B). It is worth noting that the desired effect is elicited in a mutant huntingtin context at the same concentration as that which is active in parental cells.

Compound 18 Rescues Cell Viability in HD Cell Models—We next evaluated the impact of compound 18 on $ST^{HdhQ111/HdhQ111}$ cell viability. The cells were exposed to a stress stimulus (37 °C, serum-deprived medium) in the presence or absence of nanomolar concentrations of compound 18, and cell viability was assessed by MTT assay 48 h after treatment. As shown in Fig. 5C, cell viability increased by $17 \pm 6.6\%$ in the presence of 50 nM compound 18, and an increase was observed at a dose of as little as 25 nM (Fig. 5C). This increase in cell number in the compound-treated group was not due to increased cell proliferation, because the percentage of BrdUrd-positive cells was not increased by the compound (Fig. 5D).

Compounds 2, 18, and 19 Do Not Affect REST/NRSF Translocation—We next attempted to examine $ST^{HdhQ111/HdhQ111}$ cells immunocytochemically in the presence or absence of different doses of compounds 2, 18, or 19 at different time points (4, 8, 12, 24, 48, and 72 h) using an antibody specifically recognizing the transcription factor REST/NRSF. At each time point, the nuclear and cytoplasmic levels of REST/NRSF were quantified using the Cellomics Cytoplasm to Nucleus Translocation BioApplication. This experiment did not reveal any change in the translocation of REST/NRSF induced by the treatments (data not shown), thus suggesting that other mechanisms at the RE1/NRSE sites may inhibit the activity of the silencer in HD cells exposed to the compounds.

DISCUSSION

The drug screening assay described in this report is based on a regulatory DNA sequence (the RE1/NRSE silencer within the BDNF promoter), whose silencing activity is aberrantly enhanced in HD, because increased binding of its cognate transcription factor REST/NRSF reduces the transcription of BDNF and other RE1/NRSE-controlled neuronal genes. The

expression in comparison with β -actin (-fold increase in relation to untreated cells). The data are the average of three independent experiments: **, $p < 0.01$ versus the immediately lower dose, ANOVA.

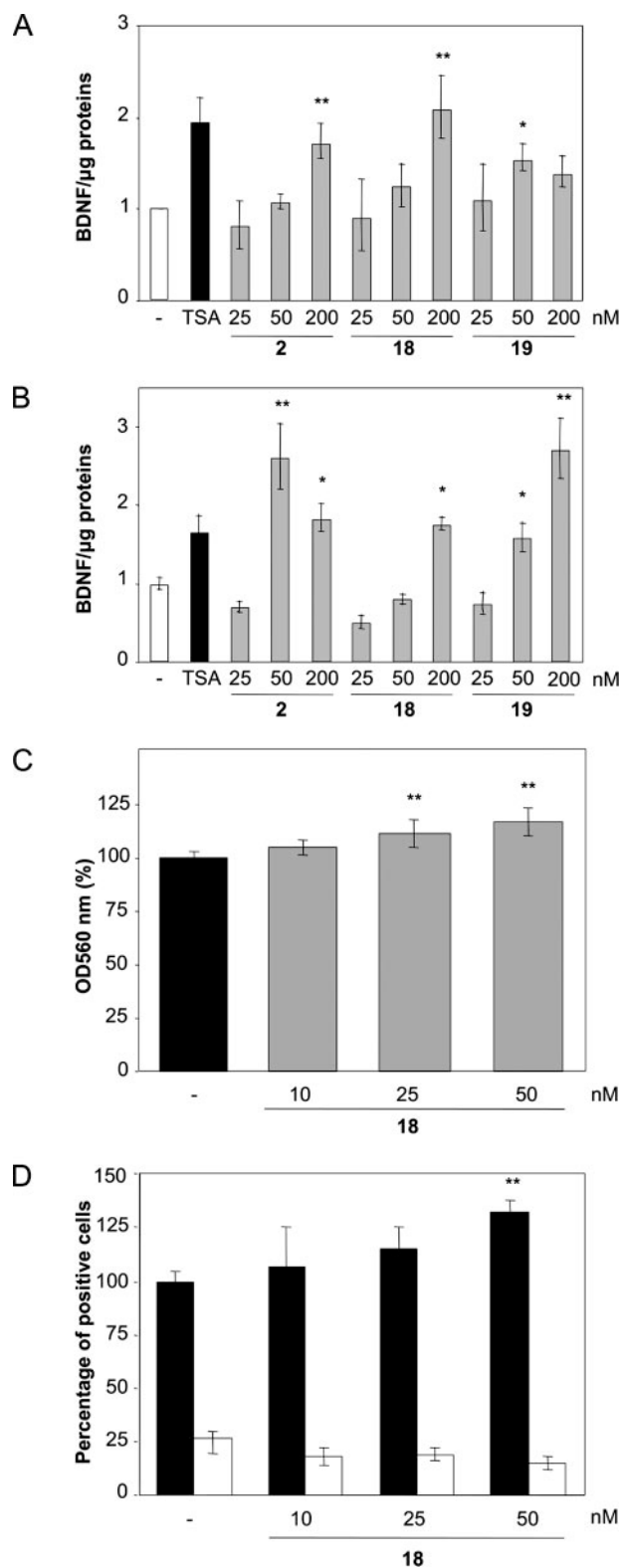


FIGURE 5. Compound administration to mutant huntingtin cells. Compounds 2, 18, and 19 were administered at the indicated concentrations to mutant huntingtin $ST^{HdhQ111/HdhQ111}$ cells (A) or neural cells stably overexpressing mutant huntingtin (B), and, 72 h later, BDNF protein levels were determined by ELISA assay (-fold increase in relation to untreated cells). The data are the average of two independent experiments. C, $ST^{HdhQ111/HdhQ111}$ cells were exposed to a stress stimulus (37 °C, serum-deprived medium) in the presence or absence of the indicated doses of compound 18, and cell viability was assessed by means of the MTT assay, 48 h after treatment. The graph shows a dose-dependent increase in cell survival in the presence of the compound.

REST/NRSF phenotype is related to reduced normal huntingtin activity, because genetic modifications of huntingtin level leading to its overexpression or depletion respectively decrease or increase REST/NRSF binding.

We have developed a stable recombinant neural cell subclone (DiaNRSE^{Luc8}) bearing a reporter construct in which luciferase activity is controlled by a 300-bp portion of the BDNF promoter containing the RE1/NRSE. The engineered cell line shows correct functional coupling of the REST/NRSF transcription factor to the RE1/NRSE^{bdnf} within the reporter construct, as assessed by the transfection of REST and its dominant negative form, and was therefore used for pilot drug screening. Three of the tested compounds proved to be capable of inhibiting the silencing activity of RE1/NRSE^{bdnf} within the reporter, thus leading to an increase in reporter activity. Remarkably, this effect occurs at low nanomolar concentrations. Moreover, a secondary assay showed that the identified compounds also inhibit RE1/NRSE^{bdnf} within the endogenous BDNF locus. The significant increase in BDNF mRNA levels is mirrored by higher BDNF protein levels and, because this effect is also elicited in a mutant huntingtin context, it is possible that the compounds may trigger the same effect in the human pathology.

Our assay was developed on the basis of a molecular mechanism that has been elucidated in HD models and confirmed in autaptic tissue (12, 13) and allows the identification of compounds that are active on RE1/NRSE-regulated genes.

The rationale for this drug-screening system, which is supported by the known positive effect of genetic or pharmacological BDNF administration in HD mouse models, is enhanced by the fact that the identified compounds may offer two advantages over BDNF administration. First, they are small molecules, which overcomes the pharmacokinetic problems usually associated with peptide administration, and, secondly (and more importantly), their effect goes beyond BDNF rescue, because they also increase the transcription of other neuronal genes bearing RE1/NRSE in their promoter, as described here in the case of synapsin I and M4 muscarinic receptors. The assay selects compounds that modulate a RE1/NRSE-controlled reporter and are, therefore, active on endogenous RE1/NRSE-regulated genes, and, because such genes are crucial for neuronal survival and phenotypic maintenance, finely regulated support of their transcription should have a positive impact on neuronal activity. This extends the application of the assay (and perhaps that of the identified compounds) to pathologies other than HD. The deregulation of REST/NRSF and its target genes in brain has in fact been demonstrated in a number of neurodegenerative conditions, including the response to ischemic stress (37, 38), Down syndrome (39), and some medulloblastomas (40). Our compounds not only increase endoge-

The data are the average of three independent experiments: **, $p < 0.01$, ANOVA. D, $ST^{HdhQ111/HdhQ111}$ cells exposed to the same experimental conditions as in C were incubated in the presence of 0.3 mg/ml BrdUrd 24 h before being fixed and then immunocytochemically processed for using an anti-BrdUrd antibody. The DNA was stained with DAPI, which confirmed an increase in cell viability in the presence of the compound (black column, percentage of DAPI-positive cells versus untreated sample). However, there were no statistically significant differences in BrdUrd incorporation (white column, percentage of BrdUrd versus DAPI-positive cells).

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nous RE1/NRSE-regulated genes and protein expression (as demonstrated in the secondary assay) but are also active in an HD cell context, in which they increase cell viability. Further medicinal chemistry studies will be performed to improve the activity *versus* toxicity ratio of the selected compound. Given the results obtained in cells, the compounds (and/or their future derivatives) will be tested in animals to verify their ability to rescue from HD molecular and phenotypic impairments.

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