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Baclofen, a $GABA_B$ receptor agonist, enhances ubiquitin-proteasome system functioning and neuronal survival in Huntington's disease model mice

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ABSTRACT

Huntington's disease (HD) is an autosomal neurodegenerative disease. Its manifestations is selective degeneration of medium-sized spiny neurons (MSN) in the striatum. The specificity of the vulnerability of these GABAergic MSNs can be explained by abnormal protein accumulation, excitotoxicity, mitochondrial dysfunction, and failure of trophic control, among other dysfunctions. In this study, we used *in vitro* and *in vivo* models of HD to study the effects of GABAergic neuron stimulation on the cellular protein degradation machinery. We administered the $GABA_B$ receptor agonist, baclofen, to wild-type or mutant huntingtin-expressing striatal cells (HD19 or HD43). Chymotrypsin-like proteasome activity and cell viability were significantly increased in the mutant huntingtin-expressing striatal cells (HD43) after $GABA_B$ receptor agonist treatment. In addition, we systemically administered baclofen to a HD model containing the entire human huntingtin gene with 128 CAG repeats (YAC128). Chymotrypsin-like proteasome activity was significantly increased in YAC128 transgenic mice after baclofen administration. Baclofen-injected mutant YAC128 mice also showed significantly reduced numbers of ubiquitin-positive neuronal intranuclear inclusions (NIIs) in the striatum. Baclofen markedly improved behavioral abnormalities in mutant YAC128 mice as determined by the rotarod performance test. These data indicate that stimulation of GABAergic neurons with the $GABA_B$ receptor agonist, baclofen, enhances ubiquitin-proteasome system (UPS) function and cell survival in *in vitro* and *in vivo* models of HD.

1. Introduction

Huntington's disease (HD) is an autosomal progressive neurodegenerative disorder involving involuntary movements associated with selective dysfunction and degeneration of the GABAergic medium-sized spiny neurons in the striatum [1,2]. HD is caused by mutations in exon 1 of the huntingtin gene (>36 CAG) [3]. Mutated huntingtin forms ubiquitin-positive neuronal intranuclear inclusions (NIIs) in the affected brain regions of HD patients [4–6] and HD model mice [7,8]. One of the potential reasons for such abnormal protein aggregation is dysfunction or downregulation of the ubiquitin-proteasome system (UPS), which plays major roles in the clearance of abnormal, misfolded, mutated, and damaged proteins in eukaryotic cells [9–11]. Supporting this model of dysfunction of the UPS, inhibited proteasome activity has been found in the striatum, frontal cortex, cerebellum, and substantia nigra of HD patients [12].

GABAergic neurons are characterized by their release of GABA (γ-aminobutyric acid) as a neurotransmitter. GABA is known to act as an inhibitory neurotransmitter in the mammalian central nervous system [13]. Synaptic inhibition by GABA can be mediated by several types of GABA receptors in the pre- and postsynaptic terminal [14]. Fast pre- and postsynaptic inhibition is mediated by $GABA_A$ receptors, which are ligand-gated ion channels. Slow pre- and postsynaptic inhibition is mediated by metabotrophic $GABA_B$ receptors, which are composed of R1a, R1b, and R2 subunits. Unlike ionotrophic $GABA_A$ receptors, metabotrophic $GABA_B$ receptors can be activated directly by GABA binding, and facilitate postsynaptic action potential [15–17]. Once activated, presynaptic $GABA_B$ receptors suppress neurotransmitter release by inhibiting Ca^{2+} channels, while postsynaptic $GABA_B$ receptors induce slow inhibitory postsynaptic current (sIPSC), activating G protein-coupled inwardly rectifying K^+ (GIRK) channels [18,19].

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In this study, we administered a specific $GABA_B$ receptor agonist, baclofen, to determine the effects of GABAergic stimulation on striatal neuronal survival and restoration of neuronal function in *in vitro* and *in vivo* models of HD.

2. Materials and methods

2.1. Cell culture and in vitro baclofen treatment

Tet-off inducible wild type (HD19, 26 CAG repeats) and mutant (HD43, 105 CAG repeats) striatal cells were cultured at 33 °C in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Hyclone), 2 mM L-glutamine (Sigma, St. Louis, MO), penicillin, and streptomycin. Expression of the huntingtin gene was induced by administration of doxycycline (1 μg/ml) for 24 h. The selective $GABA_B$ receptor agonist, baclofen (RS-baclofen; Tocris Cookson, Ellisville, MO), was administered to cultured cells at the indicated concentrations. After 24 h of administration, cell culture medium was collected for cell viability assays. Cells were harvested and lysed in a homogenization buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) containing the following protease and phosphatase inhibitors: 10 μg/ml aprotinin, 25 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml phenylmethanesulfonyl fluoride, 50 mM sodium fluoride, 50 mM sodium orthovanadate) for protein sample preparation.

2.2. Cell viability determination

We determined cell viability by performing a lactate dehydrogenase (LDH) assay on the collected cell medium, as per the manufacturer's instructions (Roche, Mannheim, Germany). LDH activities of control or baclofen-treated striatal cells were measured by absorbance at 490 nm.

2.3. In vivo baclofen administration

In this study, we used an established HD model mouse strain, a yeast artificial chromosome (YAC) mouse with the entire human huntingtin gene containing 128 CAG repeats (YAC128). Wild type (WT) and mutant (MT) male YAC128 mice at 13–18 months of age were used in this experiment. To study the effect of $GABA_B$ receptor stimulation *in vivo*, mice received intraperitoneal (i.p.) injections of baclofen (dissolved in 0.2 N NaOH) at a dosage of 10 μg/g body weight, twice daily at 9:00 a.m. and 5:00 p.m., for 3 consecutive days. Mice then received single treatments on the fourth day at 9:00 a.m., as previously described [20]. The striatum and frontal cortex tissues were collected 5 h after the last injection, and frozen until used for protein preparation.

2.4. Behavioral assessment

Approximately 16 h before the first injection and 4 h after the last injection, the mice were assessed via the rotarod test and open field test as previously described [21]. For the rotarod test, each mouse was placed on a fixed-speed rotarod (Panlab, Barcelona, Spain) at a speed of 4 rpm for a maximum time of 60 s. The latency to fall was recorded for each mouse with four trials spaced 20 min apart in a single session. The open field test was performed by placing each mouse in the center of an open-field arena (30 × 30 cm) with a light gray bottom subdivided by black lines into 6 × 6 cm squares. The 12 min session consisted of 4 min blocks, and the following activities of mice in the open field were counted during each block: 'crossings' (the number of crossings of the black lines with both forelimbs), and 'wall-rearing' (standing on hindlimbs and touching the wall of apparatus with forelimb) [22].

2.5. Immunohistochemistry

Perfused mouse brain tissues were cryoprotected and cut into 30 μm sections using a Thermo microtome (Thermo 430/Thermo Fisher Scientific, Walldorf, Germany). Immunostaining of brain sections was performed using primary antibodies against ubiquitin (1:2,000; Chemicon, Temecula, CA), followed by incubation with secondary horseradish peroxidase (HRP)-linked anti-rabbit (1:500; Jackson Laboratories, Bar Harbor, ME) or anti-mouse (1:500; Vector Laboratories, Burlingame, CA) IgG antibodies. After mounting of sections onto aminosilane-coated slides, images were obtained using a Leica fluorescence microscope (DMIL/DFC295/Leica, Bannockburn, IL).

2.6. Counting of NIIs

The number of ubiquitin-positive NIIs was counted from microscope images of three different areas of striatum in coronal sections that were subjected to the immunohistochemical procedures described above. Analysis was performed blindly by two different assessors.

2.7. Protein sample preparation and Western blot

Protein samples were purified from harvested cells and animal tissues in homogenization buffer. Lysates were centrifuged at 13,200 rpm for 30 min at 4 °C. The supernatants were collected and stored at −80 °C until use.

Cell and tissue samples containing equal amounts of total protein were analyzed by Western blot using monoclonal anti-huntingtin (1:1250; Abcam, Cambridge, UK), or polyclonal anti-proteasome β-subunit (1:5000; Calbiochem, La Jolla, CA) primary antibodies, and secondary horseradish peroxidase (HRP)-linked anti-rabbit (1:2500; Jackson Laboratories, Bar Harbor, ME) and anti-mouse (1:2500; Vector Laboratories, Burlingame, CA) IgG antibodies. Quantification of immunoreactive bands was performed by quantitative densitometry. The results were confirmed by triplicate measurements of the same sample.

2.8. Proteasome activity determination

Proteasome activity was determined by measuring the fluorescence of 7-amido-4-methylcoumarin (AMC; excitation 380 nm, emission 460 nm) generated from peptide-AMC linked substrates. Reactions were conducted in a final volume of 200 μl of buffer including 50 mM Tris–HCl (pH 7.5) and 1 mM EDTA. After adding samples to the reaction mixtures, reactions were initiated by adding the substrate Suc-Leu-Leu-Val-Try-AMC (65 μM) to measure chymotrypsin-like activity. Reactions progressed for 360 min at 25 °C. Enzymatic activity was expressed as fluorescence units (FU)/mg/min of protein.

2.9. Statistical analysis

All statistical analyses were performed using the SPSS software package (version 12.0.1; SPSS Inc., Chicago, IL). Data were objectively compared between different groups of cells or mice using the independent-samples *T*-test. Differences between groups were considered statistically significant when the p value was less than 0.05.

3. Results

3.1. Baclofen enhances chymotrypsin-like proteasome activity and cell viability in HD model cells

To address the effect of GABAergic stimulation on proteasome activity in HD, we measured chymotrypsin-like proteasome activity in HD model striatal cells, HD19 (26 CAG repeats) and HD43 (105 CAG repeats), after baclofen treatment. HD43 cells showed a significant increase in chymotrypsin-like proteasome activity at 10 μM of baclofen, whereas HD19 cells did not exhibit significant changes (Fig. 1A). To determine whether GABAergic stimulation also increases the viability of HD model cells, we measured LDH activity as an indicator of cell viability. As shown in Fig. 1B, the culture media from HD43 cells had markedly decreased LDH activity, indicating increased cell viability, after treatment with baclofen at 1 or 10 μM. These data indicate that stimulation of GABAergic neurons with a specific $GABA_B$ receptor agonist can increase chymotrypsin-like proteasome activity and cell survival in mutant HD model cells.

3.2. Baclofen increases proteasome activity and reduces NIIs in YAC128 HD transgenic mice

To verify whether the improvements in proteasome activity and cell survival also occur *in vivo*, we administered 10 μg of baclofen per gram of body weight to YAC128 HD transgenic mice. Chymotrypsin-like proteasome activity decreased in the striatum of mutant type (MT) YAC128 mice compared to wild type (WT) littermates. This decreased proteasome activity in the striatum of mutant YAC128 mice is also seen in human HD patients [12] and mutant YAC72 mice [23]. Corresponding to the *in vitro* data, chymotrypsin-like proteasome activity significantly increased in the striatum of MT YAC128 mice after baclofen administration (Fig. 2A). In addition, the expression level of the 20S proteasome β-subunit significantly increased in the striatum of both WT and MT YAC128 mice after baclofen administration (Fig. 2B).

Next, to confirm whether the increased proteasome activity effectively degrades polyglutamine aggregates in the affected region of the brain, we counted the number of ubiquitin-positive NIIs in the striatum of WT and MT YAC128 mice after baclofen administration, by immunohistochemical analysis. As shown in Fig. 2C, ubiquitin-positive NIIs were detected in the striatum of WT and MT YAC128 mice after baclofen administration. Statistical analysis of the number of NIIs indicated that baclofen-injected mutant YAC128 mice showed significantly reduced NIIs formation compared to vehicle-injected mutant YAC128 mice (Fig. 2C). These data suggest that GABAergic stimulation ameliorates pathological symptoms of HD by diminishing NIIs in the striatum.

3.3. Baclofen ameliorates motor deficits in YAC128 HD transgenic mice

Finally, we assessed whether behavioral abnormalities in YAC128 HD mice are improved by baclofen administration. Compared to WT littermates, MT YAC128 mice showed significantly reduced latency to fall (Fig. 3) in the rotarod test and number of crossing in open-field test, as had been observed in a previous study [7]. There were no differences in the number of wall-rearing episodes between WT and MT YAC128 mice. Baclofen administration significantly increased the latency to fall in the rotarod test in mutant YAC128 mice (Fig. 3), Total locomotor counts of crossing and frequency of wall-rearing episodes were not significantly altered in MT YAC128 mice after baclofen administration (Fig. 3). These data demonstrate that the $GABA_B$ receptor agonist restored motor coordination in YAC128 HD mice.

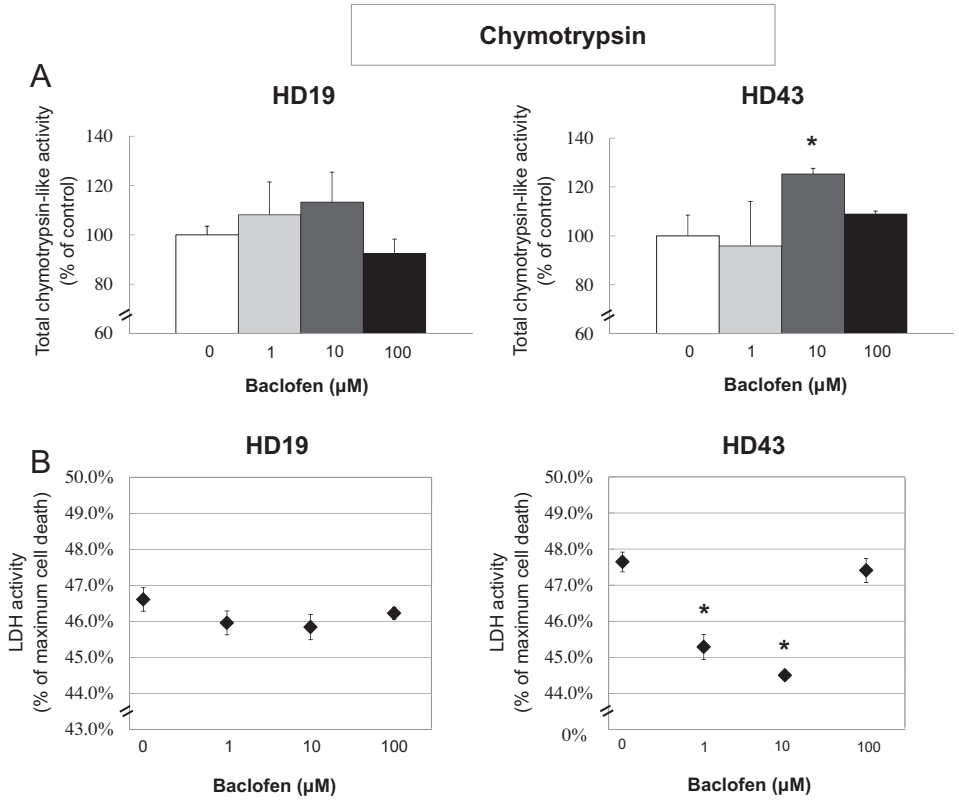

Fig. 1. The effect of baclofen on proteasome activity and cell viability in an *in vitro* HD model. (A) Chymotrypsin-like proteasome activity was significantly increased in mutant (HD43, 105 CAG repeats) striatal neurons at a baclofen concentration of 10 μM. (B) Cell viabilities of baclofen-treated HD19 and HD43 cells were determined by LDH assay. HD43 cells showed a significant increase of cell viability following 1 μM and 10 μM baclofen treatment (*$p < 0.05$).

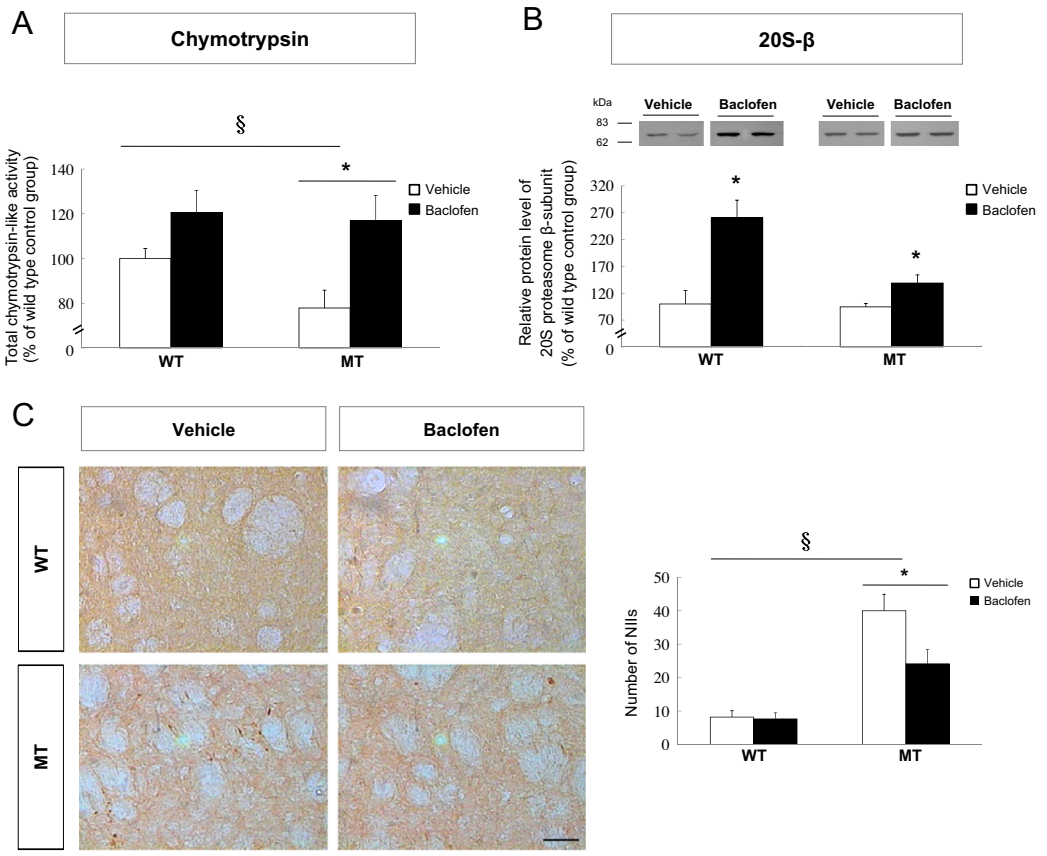

Fig. 2. The effect of baclofen on proteasome activity and inclusion bodies in the striatum of YAC128 HD transgenic mice. (A) Mutant (MT) YAC128 mice showed significantly decreased chymotrypsin-like proteasome activity in the striatum compared to wild type (WT) littermates. Chymotrypsin-like proteasome activity was significantly increased in the striatum of MT YAC128 mice following baclofen administration. (B) The expression levels of the 20S proteasome β-subunit significantly increased in the striatum of both WT and MT YAC128 mice ($^{*}p < 0.05$, $^{**}p < 0.01$). (C) Ubiquitin positive NIIs were detected by immunohistochemistry using anti-ubiquitin antibody, in the striatum of WT or MT YAC128 mice after baclofen administration. The number of ubiquitin-positive NIIs was significantly reduced in the striatum of baclofen-injected mutant YAC128 mice, compared to vehicle-injected group ($^{*}p < 0.05$ within wild type or mutant group, $^{\S}p < 0.05$ between wild type and mutant groups). Scale bar indicates 50 μm.

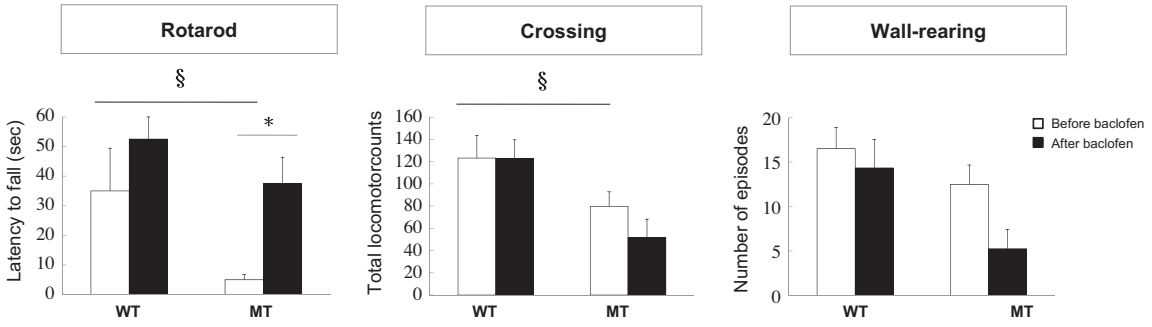

Fig. 3. The effects of baclofen on motor behavior of MT YAC128 HD mice. MT YAC128 mice showed decreased latency to fall compared to WT littermates. After baclofen administration, MT YAC128 mice showed significantly improved latency to fall in the rotarod test. Total locomotor counts of crossing and frequency of wall-rearing episodes showed tendency to decrease in MT YAC128 mice after baclofen administration ($^{*}p < 0.05$ within wild type or mutant group, $^{\S}p < 0.05$ between wild type and mutant groups).

4. Discussion

4.1. GABA and $GABA_B$ receptor expression in HD

A hallmark of HD is selective cell death of the GABAergic medium-sized spiny neurons in the striatum. The ubiquitin-positive nuclear inclusion bodies of mutant huntingtin observed in the affected brain regions of HD patients are due to downregulation of UPS function. In this study we aimed to enhance GABAergic neuronal activation and proteasome function to overcome HD pathogenesis.

The expression levels of $GABA_B$ receptors are not altered in the affected brain regions in HD model rodents [24–26], but GABA immunoreactivity is markedly reduced in the striatum of quinolinic acid (QA)-induced HD model rats [27]. High-resolution magic angle spinning (HR-MAS) ^{1}H nuclear magnetic resonance (NMR) spectroscopy revealed that GABA concentrations are markedly reduced in the striatum and cerebellum of the R6/2 transgenic mouse model of HD [28]. In human HD patients, cerebrospinal fluid (CSF) GABA levels are quite low [29]. Baclofen, a specific $GABA_B$ receptor agonist, mimics the action of GABA and produces slow presynaptic inhibition through the $GABA_B$ receptor [30–32]. Owing to this

effect of presynaptic inhibition, baclofen is employed in diseases that are accompanied by spasm or convulsion. Intraperitoneal injection of baclofen reduced audiogenic seizures in fragile X mental retardation protein (FMR1) knockout mice [33]. Intrathecal baclofen treatment results in improvement of dystonia of the hands in human patients with reflex sympathetic dystonia [34]. Accordingly, in this study we hypothesized that functional activation of $GABA_B$ receptors by baclofen could stimulate GABAergic medium-sized spiny neurons, and ameliorate the involuntary and uncoordinated behavioral pathology of HD.

4.2. The effect of baclofen on regulation of proteasome activity and NIIs formation

To determine whether functional stimulation of GABAergic MSNs through $GABA_B$ receptor activation alters ubiquitin-proteasome system function, we measured proteasome activities and the protein levels of the proteasome core 20S unit from WT and MT YAC128 mice after baclofen administration. YAC128 HD transgenic mice exhibit selective loss of MSNs in the striatum at 9 months of age [7]. In the current study, chymotrypsin-like activity decreased in the striatum of MT YAC128 mice, similar to previous reports of human HD patients and MT YAC72 HD model mice [12,23]. Baclofen administration increased chymotrypsin-like activity and the survival of MSNs in the striatum of MT YAC128 mice.

Previous studies have reported that NIIs formation in polyglutamine diseases is closely related to reduced proteasome activity [35–37] and that ubiquitin-positive NIIs result in apoptotic cell death in mutant huntingtin-transfected neuroblastoma [38]. In the current study, we determined that baclofen significantly decreased NIIs formation in the striatum of YAC128 mice. This result suggests that $GABA_B$ receptor activation effectively improves proteasome function, and thus significantly reduces pathological NIIs in the striatum of HD model mice.

4.3. The effect of baclofen on motor coordination of mutant YAC128 mice

Motor dysfunction of mutant YAC128 mice correlating with striatal neuronal loss begins at 6 months of age, as determined by fixed speed rotarod performance [7]. The latency to fall of mutant YAC128 mice significantly increased after baclofen administration. In an open field test, MT YAC128 mice exhibited decreased activity of locomotion compared to WT littermates, as previously reported [7,39]. In this study, baclofen administration showed tendency to decrease the locomotive activities of crossing and wall-rearing in MT YAC128 mice. These data suggest that a specific $GABA_B$ receptor agonist can improve behavioral abnormalities associated with motor coordination.

In summary, functional GABAergic stimulation via a $GABA_B$ receptor enhances survival of striatal cells and proteasome activity *in vitro*, reduces ubiquitin-positive NIIs, and consequently induces restoration of motor function in *in vivo* models of HD. Our data suggest that $GABA_B$ receptor agonists could be promising therapeutic reagents for the treatment of HD.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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