Elucidating the role of the A2A adenosine receptor in neurodegeneration using neurons derived from Huntington’s disease iPSCs

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Abstract

Huntington’s disease (HD) is an autosomal-dominant degenerative disease caused by a cytosine-adenine-guanine trinucleotide expansion in the Huntingtin (htt) gene. The most vulnerable brain areas to mutant HTT-evoked toxicity are the striatum and cortex. In spite of the extensive efforts that have been devoted to the characterization of HD pathogenesis, no disease-modifying therapy for HD is currently available. The A2A adenosine receptor (A2AR) is widely distributed in the brain, with the highest level observed in the striatum. We previously reported that stimulation of the A2AR triggers an anti-apoptotic effect in a rat neuron-like cell line (PC12). Using a transgenic mouse model (R6/2) of HD, we demonstrated that A2AR-selective agonists effectively ameliorate several major symptoms of HD. In the present study, we show that human iPSCs can be successfully induced to differentiate into DARPP32-positive, GABAergic neurons which express the A2AR in a similar manner to striatal medium spiny neurons. When compared with those derived from control subjects (CON-iPSCs), these HD-iPSC-derived neurons exhibited a higher DNA damage response, based on the observed expression of γH2AX and elevated oxidative stress. This is a critical observation, because oxidative damage and abnormal DNA damage/repair have been reported in HD patients. Most importantly, stimulation of the A2AR using selective agonists reduced DNA damage and oxidative stress-induced apoptosis in HD-iPSC-derived neurons through a cAMP/PKA-dependent pathway. These findings support our hypothesis that human neurons derived from diseased iPSCs might serve as an important platform to investigate the beneficial effects and underlying mechanisms of A2AR drugs.
Introduction

Huntington’s disease (HD) is an inherited, autosomal-dominant, progressive neurodegenerative disease, caused by abnormal expansion of cytosine-adenine-guanine (CAG) repeats in exon1 of the huntingtin (htt) gene. CGG repeats in excess of 36 result in the production of mutated huntingtin (HTT) proteins with an abnormally long polyglutamine stretch; these proteins form aggregates in the cytoplasm and nucleus, which cause degradation of neurons, particularly GABAergic medium-sized spiny projection neurons (MSNs) in the striatum. MSNs are involved in the regulation of movement, emotion and cognitive ability (1,2), and thus HD patients gradually suffer from increased irritability and depression, involuntary movements (chorea), rigidity and eventually dementia. Although the mutation which causes HD is known, our understanding of HD pathogenesis remains poor. Moreover, there is currently no effective strategy to delay the onset or slow down the progression of HD.

Various models have been developed to facilitate investigation into disease pathogenesis and to develop therapeutics. However, most studies of HD have utilized transgenic mice or immortalized neurons carrying the mutant htt gene (3), and these models may not fully recapitulate human HD. Recent advances in human-induced pluripotent stem cell (iPSC) technology have enabled the generation of patient-specific iPSC lines for studying disease pathogenesis and for use as a drug screening platform (4,5). Multiple research groups have isolated several human HD-iPSCs, and induced their differentiation into a disease-relevant cell type [DARPP-32-positive cells, which are expressed in the majority of MSNs (6)]; however, the differentiation efficiency was low (5–10%) (7–9). Increasing such efficiency is important for the effective study of disease pathogenesis.

Cell-autonomous events contribute to mutant HTT-induced toxicity in affected neurons in mammalian cell culture systems and transgenic mouse models of HD. Several studies have suggested that multiple mechanisms may underlie the neurotoxic effect of mutant HTT, including excitotoxicity (10,11), oxidative stress and mitochondrial abnormalities (10,12), defects in calcium homeostasis (13,14), ubiquitin-proteasome system impairments (15,16) and transcriptional dysregulation (12,17). However, such evidence is insufficient to account for the selective vulnerability of neurons, because mutant HTT is ubiquitously expressed in the brain and body. Consequently, these studies imply that additional components are involved in neuronal cell death in HD.

Adenosine is a well-characterized regulator of neuronal survival, which exerts its function through binding to specific cell surface adenosine receptors. To date, four adenosine receptor subtypes, A1, A2A, A2B and A3, have been identified. The A2A adenosine receptor (A2AR) is a Gαs-coupled receptor. Upon stimulation, the A2AR activates multiple classical signaling pathways, including the production of cAMP that activates PKA (18,19). The A2AR exhibits differential distribution within the brain, with the highest expression in the encephalin-expressing MSN of the striatum (20,21). Expression of this receptor is affected by mutant HTT, and is gradually down-regulated at the transcriptional level during the progression of HD (22–24). Nonetheless, the ability of the A2AR to enhance the cAMP level in the brain of HD mice is similar to that of WT mice (23), suggesting that A2AR signaling in HD is amplified. Thus, the A2AR remains a potential drug target of HD (25). This possibility is of great interest because it has been shown that tonic activation of the A2AR is required for the function of several neurotrophic factors, including brain-derived neurotrophic factor, fibroblast growth factor and glial cell line-derived neurotrophic factor (26–28). In genetic mouse models of HD, accumulating evidence indicates that stimulation of the A2AR may promote neuronal survival. For example, it has been shown that an A2AR agonist (CGS21680) can (i) significantly delay deterioration of motor coordination (23), (ii) increase proteasome activity and reduce the accumulation of mutant Htt aggregates (23,29) and (iii) attenuate NMDA-induced toxicity in corticostriatal slices of a transgenic mouse model of HD (R6/2) (30). Accordingly, A2AR-related drugs have been implicated as possible treatments for human HD.

In this study, we generated and characterized human HD-iPSCs with a CAG expansion mutation in the htt gene, and induced their differentiation into GABAergic and DARPP-32-positive neurons. Consistent with previous reports (8,9,31,32), we did not observe mutant HTT aggregation in neurons derived from HD-iPSCs. Nevertheless, we observed selective vulnerability of HD-iPSC-derived neurons to oxidative stress, which is an important feature of HD pathology (33–35). Furthermore, we demonstrated that activation of the A2AR by selective agonists (CGS21680 and APEC) reduced oxidative stress-induced DNA damage and caspase 3 activation in the HD-iPSC-derived GABAergic neurons. Our data imply that the human HD-iPSC model recapitulates, at least in part, the HD disease phenotype in vitro, and thus may be a potential platform for drug screening.

Results

Derivation and characterization of HD iPSCs

To generate iPSCs, fibroblasts or peripheral blood mononuclear cells obtained from four non-HD controls and two patients diagnosed with HD were transduced with five reprogramming factors, namely OCT4, SOX2, KLF4, c-MYC and NANOG (OSKM) (Supplementary Material, Table S1). Following 2–3 weeks under human embryonic stem cell (hESC) culture conditions, several compact, refractive, ESC-like colonies began to emerge. Four to six iPSC lines were isolated for each subject and characterized by various assays. Specifically, the integration of exogenous OSKMN transgenes into the genomes of the HD-iPSC clones was confirmed by PCR analysis at passage six (Fig. 1A). RT-PCR analysis of the HD-iPSC lines revealed reactivation and expression of the endogenous pluripotency-associated markers, including OCT4, SOX2, NANOG, KLF4 and c-MYC, whereas the exogenous OSKMN transgenes were found to be predominantly silenced (Fig. 1B).

PCR analysis of genomic DNA was also used to show that HD-iPSC-A1 and HD-iPSC-B4 contained mutant htt overlap with CAG expansion (both with 43 repeats), as also observed for their parental HD fibroblasts (Fig. 1A and C). In contrast, non-HD control iPSCs (CON-iPSCs) contained only normal htt alleles with shorter CAG triple repeats. Immunocytochemical (ICC) analysis with antibodies against ESC antigens was used to demonstrate that HD-iPSCs express pluripotency-associated genes and markers without losing their pluripotency characteristics after extended culture in vitro (Fig. 1D). The in vivo developmental propensity of HD-iPSCs was examined using the teratoma formation assay. Following intramuscular injection of undifferentiated HD-iPSCs into immunocompromised mice, teratomas consisting of cell types representing all three embryonic germ layers were formed (Fig. 1E).

Differentiation of HD-iPSCs into disease-relevant neuronal subtypes

We proceeded to investigate the neuronal differentiation potential of the HD-iPSCs. As shown in Figure 2A, HD-iPSCs were...
subjected to embryoid body (EB) formation followed by step-wise in vitro differentiation. Following replating of EBs onto a cell culture surface in N2 media containing 20 ng/ml bFGF, rosette-like structures resembling early neuroepithelium and neural progenitors began to emerge from EB outgrowths. ICC staining and RT-PCR analysis were used to demonstrate that these cells express neural progenitor markers, such as Nestin, SOX1 and PAX6, but not the ESC markers OCT4 or Nanog (Fig. 2B and C). Further culture of the neural progenitors in N2/B27 media with 10 ng/ml bFGF directed their differentiation into various neural populations, including TUJ1-expressing or MAP2-expressing neurons, and Olig2-expressing or GFAP-expressing glia cells (Fig. 2B and C). In addition to expression of neural markers, we also detected the mHTT transcript in iPSC-derived neurons.

RT-PCR was used to show that HD-iPSC neurons contained mutant htt with CAG expansion, while CON-iPSC neurons contained only normal htt with shorter CAG triple repeats (Fig. 2D).

Since GABAergic projection neurons are the primary neuronal subtype affected in HD brains, we proceeded to examine the proportion of GABAergic neurons in our model. To enhance neuronal differentiation, we cultured the differentiated neural progenitors in B27 media for an additional 4–6 weeks after 4 weeks of culture in B27/N2 media. Next, we interrogated the differentiated cells at various developmental stages, using GABAergic neuron markers, including γ-aminobutyric acid (GABA), Glutamate decarboxylase (GAD) 65, Calbindin and dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32). We observed that cells at the early stages of differentiation

Figure 1. Characterization of human HD-iPSC cell lines. (A) Genomic PCR was used to show that all HD-iPSC lines in this study possessed integrated viral OCT4, SOX2, KLF4, c-MYC and NANOG (OSKM) genes. (B) Semi-quantitative PCR analysis was used to show that the viral OSKM exogenous genes (exo) were silenced and endogenous loci (end) of OSKM were reactivated in these HD-iPSC lines. (C) Genomic PCR and sequencing confirmed the CAG expansion mutation. The H9 hESC line (hESC), normal human foreskin fibroblasts (CON-HF) and HD dermal fibroblasts from HD patients (HD-A/B-HF) were used as controls. β-actin and GAPDH were used as loading controls. (D) Phase contrast and ICC analysis of pluripotency markers (OCT4, SOX2, Nanog and SSEA4) in HD-iPSC lines (A1, A7, B4 and B16). (E) Hematoxylin and eosin staining of tissue sections of teratomas derived from two HD-iPSC lines (A1 and B4) grafted into immunodeficient mice. Scale bar: 50 μm.
already expressed GABA, while some even expressed the striatal neuron markers GAD65 and Calbindin; cells expressing DARPP-32, a MSN marker, were observed rarely under this condition (data not shown). At later stages of differentiation, the majority of cells were process-bearing neurons that expressed TUJ1 and MAP2; in addition, a high proportion of cells at this stage (over 70%) were positive for GABAergic projection neuron markers: GABA, GAD65, DARPP-32, or Calbindin (Fig. 3A–D, Supplementary Material, Fig. S1B and Supplementary Material, Table S5). We assessed the homogeneity of the neuronal populations derived from CON- or HD-iPSCs. First, we examined viable cells by Trypan blue exclusion assay at weeks 4, 8 and 12 of neural differentiation; this revealed that the expansion rates are similar between CON- and HD-iPSC-derived NPCs (Supplementary Material, Fig. S1A). In addition, the neuronal populations derived from CON- and HD-iPSCs exhibit similar homogeneity, as revealed by ICC, Western blotting and RT-qPCR analysis (Fig. 3D and E, Supplementary Material, Fig. S1B–C and S2). Together, these results indicate that the majority of neuronal cells derived from HD-iPSCs or CON-iPSCs are GABAergic projection neurons, and that the neural differentiation efficiencies are similar between CON-iPSC- and HD-iPSC-derived NPCs.

**HD-iPSC neurons are more susceptible to H2O2-induced DNA damage than controls**

Increasing evidence indicates that oxidative stress plays an important role in HD pathogenesis (33–35). To examine whether HD-iPSC-derived neurons are vulnerable to oxidative stress-induced DNA damage, we treated neurons derived from both HD- and CON-iPSCs with H2O2. ICC analysis and immunoblotting with an antibody against γH2AX, as a DNA damage marker (36), were used to show that H2O2 induced γH2AX activation in a time- and dose-dependent manner in neurons derived from both HD- and CON-iPSCs (Fig. 4 and Supplementary Material, S3); this suggests that oxidative insult promotes DNA double-strand breaks (DSBs) in the iPS-induced neurons. However, H2O2 induced a significantly greater level of γH2AX in HD-iPSC-derived
neurons (Fig. 4C–E). We also compared the global gene expression profiles of HD- and CON-iPSC-derived neurons by microarray analysis (Supplementary Material, Table S6), which revealed that genes encoding proteins involved in the repair of DNA DSBs (RAD51) and protection against oxidative stress (GSTT1, GSTT2) were down-regulated in HD-iPSC-derived neurons. Collectively, these data indicate that HD-iPSC neurons are more susceptible to H2O2-induced DNA damage.

Activation of the A2AR protects HD-iPSC neurons from H2O2-induced DNA damage and apoptosis through a PKA-dependent pathway

Activation of the A2AR has been previously demonstrated to have several beneficial effects in genetic mouse models of HD (23,29,37). To gain a better understanding of how the A2AR exerts its neuroprotective effect, we first examined the expression of the A2AR in HD-iPSC-derived neurons. ICC analysis, RT-qPCR and immunoblotting were used to demonstrate that iPSC-derived neurons express the A2AR (Fig. 5), and that HD-iPSC-derived neurons express a lower level of the A2AR than control iPSC-derived neurons. Collectively, these data indicate that HD-iPSC neurons are more susceptible to H2O2-induced DNA damage.

Activating the A2AR by CGS-21680 or APEC significantly reduced the H2O2-induced γH2AX activation in HD-iPSC-derived neurons in a dose-dependent manner. We also confirmed that CGS has the same protective effect in CON-iPSC-derived neurons (Supplementary Material, Fig. S4C and D). To investigate whether the HD-iPSC-derived neurons are susceptible to cell death upon H2O2 exposure, we treated HD- and CON-iPSC-derived neurons with H2O2, and performed TUNEL assays and measured active caspase 3 to quantify the percentage of cells with apoptotic traits. We observed that H2O2 treatment resulted in a greater enhancement of active caspase 3 in HD-iPSC-derived neurons than in their counterparts derived from CON-iPSCs (Fig. 6C, D and F). Consistent with the effects on caspase-3 activation, H2O2 treatment resulted in a significantly higher proportion of TUNEL-positive HD-iPSC-derived neurons than TUNEL-positive CON-iPSC-derived neurons. Addition of CGS reduced the TUNEL-positive cells to ~10% (Fig. 7G).
To determine the basis for the protective effect of A2AR agonist on H2O2-induced DNA damage and apoptosis, we used immunoblotting to examine induction of γH2AX and cleaved capase-3 in control-iPSC neurons transfected with A2AR-shRNA or control-shRNA. A2AR protein was readily detected in cells transfected with a scrambled control shRNA, while the level of expression was significantly reduced in cells transfected with the A2AR shRNA (Supplementary Material, Fig. S5A). As shown in Supplementary Material, Figure S5B and S5C, treatment with CGS decreased the H2O2-mediated induction of γH2AX and cleaved caspase-3 in control-shRNA transfectants. In contrast, the protective effect was blocked in A2AR-shRNA transfectants. These findings support the conclusion that A2AR activation plays an important role in H2O2-induced DNA damage and apoptosis.

As it has been previously shown that A2AR activation prevents toxic stimuli-induced apoptosis through PKA activation (38, 39), we hypothesized that PKA activation may be involved in the A2AR-mediated protection of HD-iPSC-derived neurons. Hence, we pre-treated HD-iPSC-derived neurons with an A2AR antagonist (SCH58261) and a selective PKA inhibitor (H-89) prior to H2O2 and CGS exposure, and subsequently examined the extent of DNA damage (γH2AX) and apoptosis (TUNEL and caspase 3 activation). As demonstrated by ICC analysis and immunoblotting, we found that the protective effect of CGS against both H2O2-induced DNA damage (Fig. 7A, B and E) and cell death (Fig. 7C, D, F and G) can be abolished by treatment with SCH58261 or H-89. Together, these data indicate that activation of the A2AR exerts a beneficial effect on HD-iPSC-derived neurons.
neurons, protecting them from DNA damage and cell death via activation of PKA.

**Discussion**

Here, we describe the generation of human HD-iPSCs with a CAG expansion mutation in the endogenous htt gene, and their differentiation into HD-relevant, DARPP-32-positive, GABAergic neurons. We confirmed that these cells exhibit selective vulnerability to oxidative stress, which is an important feature of HD pathology. Using these HD-iPSC-derived neurons as an in vitro model, we have demonstrated that activation of the A2AR by two A2AR-selective agonists (CGS and APEC) can reduce oxidative stress-induced DNA damage and neuronal apoptosis in HD-iPSC-derived neurons, through a PKA-dependent pathway. This study demonstrates the feasibility of using human-iPSC-derived MSN neurons as a drug screening platform for HD. Importantly, the CAG repeat number in the htt gene of our HD-iPSCs is 43, which is in the range for most HD patients, and is much lower than that of previously described HD-iPSCs (8,31). Thus, the results obtained from our HD-iPSC cells are likely to recapitulate HD pathology observed in the majority of HD patients. Collectively, our findings suggest that the human HD-iPSC model recapitulates HD-relevant pathogenesis, and may thus be suitable for further development into a platform for screening novel therapeutic strategies.

Human iPSC-based models for HD have been developed to replicate specific molecular and biological phenotypes. However, consistent with previous reports (8,9,31,32), we did not observe mutant HTT aggregation, an overt HD phenotype, in neurons derived from HD-iPSCs. Recently, human HD iPSC lines were used to derive neuronal precursors; these precursors exhibited a mild increase in caspase activity upon growth factor deprivation (9,31), and lysosomal activity was significantly increased in HD-iPSCs and HD-iPSC-derived neurons as compared with their CON-iPSC counterparts (32). The HD-iPSC consortium and Carter et al. (8,40) reported that HD-iPSC differentiated cultures were highly susceptible to stressors, including BDNF withdrawal, glutamate pulsing, or the addition of cellular stressors (H2O2 and 3-MA). Our results demonstrate that oxidative stress is involved in the pathogenesis of HD; HD-iPSC-derived neurons were more susceptible to H2O2-induced DNA damage and cell death than their counterparts derived from normal iPSCs. These results are consistent with previous reports that H2O2 treatment resulted in a strong DNA damage response in fibroblasts derived from HD patients (41), and increased cell death in human HD iPSCs (8,40). It has also been suggested that the association between HD and increased ROS and oxidative stimuli (8,42) underlies mutant htt-dependent cell death (8,43).

Recent evidence indicates that oxidative stress-induced defects in DNA damage repair can lead to neuronal cell death (33,44,45). Our microarray analysis data (Supplementary Material, Table S6) revealed that expression of RAD51, which is involved in recombination and repair of damaged DNA, is reduced in HD-iPSC neuron derivatives as compared with counterparts from CON-iPSCs. We also observed that expression of genes encoding cellular glutathione antioxidant enzymes [glutathione S-transferase theta 1 and 2 (GSTT1, GSTT2) and Glutathione peroxidase 8 (GPX-8)] was slightly decreased in HD-iPSC-derived neurons (Supplementary Material, Table S6). These results are consistent with the reported increase in oxidative stress biomarkers and reduction in antioxidant systems in HD patients (42). Despite the presence of such defects, we did not observe increased mortality under basal neuronal culture conditions in the long term. Nevertheless, these changes may increase the sensitivity of HD-iPSC-derived neurons to oxidative stress, thereby resulting in the accumulation of ROS, and consequent DNA damage and cell death. Moreover, our results revealed that one of the protective functions of the A2AR in human MSN neurons derived from HD-iPSCs is the prevention of DNA damage...
evoked by elevated oxidative stress. This new finding provides important insights into the function of the A2AR as a potential therapeutic target for HD in human neurons.

Although the gene causing HD is known, the mechanism underlying the increased vulnerability of a specific neuronal population in HD remains elusive. Previous studies have suggested a role of the A2AR in cell survival. For example, stimulation of the A2AR has been shown to protect against cell death induced by hypoxia (46) and serum-deprivation (39,47) in PC12 cells, and NGF withdrawal in sympathetic neurons (48). Our current findings demonstrate that CGS and APEC prevent DNA damage and apoptosis induced by H2O2 in HD-iPSC-derived neurons (Figs 6 and 7), suggesting that the A2AR exerts a positive effect on the survival of human HD-iPSC-derived neurons. Additionally, we confirmed the protective effect of an A2AR agonist on H2O2-induced DNA damage and apoptosis by shRNA knockdown of...
the A2A-R. Our results demonstrated that CGS treatment decreased DNA damage and apoptosis induced by H2O2 insult in control-shRNA transfectants, whereas the protective effect was blocked in A2A-R-shRNA transfectants (Supplementary Material, Fig. S5B and S5C). These findings further support the conclusion that A2A-R activation plays an important role in H2O2-induced DNA damage and apoptosis. Our findings are consistent with previous reports, which showed that A2A-R agonists are beneficial in a

Figure 7. The protective effects of A2A-R agonists are mediated through activation of PKA. The HD-iPSC-derived neurons were pre-treated with SCH58261 (SCH, 10 μM) or H-89 (H89, 10 μM) and then treated with CGS21680 (CGS, 10 μM) and 100 μM H2O2 for 1 h (DNA damage) or 6 h (cell death), respectively. Immunofluorescence analysis of neurons stained with (A) γH2AX and TUJ1 or (C) active caspase-3 and TUJ1. Nuclei were stained with DAPI. Scale bar: 20 μm. Quantitative analysis of the intensity of (B) γH2AX and (D) active caspase-3, as normalized to total cell number by counting DAPI-labeled nuclei. At least 500 cells were quantified for each condition of each experiment. Immunoblotting of neurons with antibodies against (E) γH2AX and (F) active caspase 3. GAPDH was used as a protein loading control. Histograms correspond with the lanes at the top of the adjacent panel (from left to right). Data are representative of three independent experiments. (G) Percentage of TUNEL-positive cells within total cells. Data are presented as the mean ± SEM of three independent experiments. At least 200 cells were quantified for each condition of each experiment. *, P < 0.05 [by one-way ANOVA in (B) and (D); by Student’s t test in (E), (F) and (G)].
transgenic mouse model of HD (R6/2) and mouse striatal progenitor cell lines (HdhTg and Hdh109Q) (23,37,49–51). In line with the aforementioned observations supportive of a protective role for the A2AR in human and rodent models of HD, genetic removal of the A2AR significantly exacerbates the progression of HD in a

HD mouse model (N171-82Q) (52). Together, these findings strongly suggest that the A2AR is a worthwhile drug target for HD.

Stimulation of the A2AR activates at least two major cellular signaling cascades: adenyl cyclase/CAMP/PKA and PKC-mediated pathways (19). Several reports have indicated that components involved in regulating the PKA pathway are disrupted during HD progression (22,53,54). Therefore, the role of PKA in the protective function of the A2AR is worthy of further evaluation.

A2AR-mediated protection against H2O2-induced DNA damage and cell death in HD-iPSC-derived neurons appears to be mediated by PKA, because such protection was blocked by a PKA inhibitor, H-89 (Fig. 7). Our results are consistent with earlier reports, which demonstrated that A2AR activation protects cells against hypoxia and growth factor withdrawal via PKA signaling (39,46,55).

The ability of iPSCs to differentiate into GABAergic MSNs, the most susceptible cell type in HD pathogenesis, will be the crux to successfully model HD in culture. Procedures for inducing differentiation of hESCs and iPSCs into GABAergic neurons have been described previously (7–9,56,57). These protocols often involved treatment with various morphogens, such as SHH and WNT inhibitor (DKK1), to direct hESC differentiation toward the GABAergic neuronal lineage (7–9,57). By using a step-wise in vitro differentiation protocol combining EB formation, neural induction by small molecules, treatment with inhibitors of the TGFβ pathway (SB431542) and the BMP pathway (LDN193189) (58), and mechanical isolation/purification of neural progenitors and neurons, we induced 60–70% of control iPSCs or HD-iPSCs to differentiate into GABA- and DARPP-32-double positive neurons (Fig. 3D). The high yield of GABA- and DARPP-32-double positive neurons obtained may be attributed to effective neural induction by the BMP and TGFβ inhibitors and the mechanical isolation/purification of neural progenitors and early neural cells. This is of particular interest because the default neuronal differentiation of human expanded neuron precursors typically produces GABAergic neurons, irrespective of the region from which these cells were harvested from the embryo (59,60).

In summary, we have elucidated the role of the A2AR in HD using HD-iPSC-derived neurons as a disease modeling system. Our results imply that A2AR activation may protect MSN against ROS-induced damage, and suggest that the A2AR may be a novel therapeutic target for HD.

Materials and Methods

Reagents

SB431542, 2-[p-(2-carboxyethyl) phenylethylamino]-5′-N-ethylcarboxamidoadenosine [CGS21680, (61)], and 5-amino-7-(2-phe- nylyl)-2-(2-furyl)-pyrazolo[4,3-c]-1,2,4-triazolo[1,5-c) pyrimidine [SCHS8261, (62)] were purchased from Sigma Aldrich (St Louis, MO, USA). Adenosine deaminase (ADA) (1 unit/ml) for 4 h to eliminate endogenous adenosine, and then treated with an A2AR agonist (CGS21680 or APEC) of the indicated concentration for 1 h, prior to the addition of H2O2 (100 mM). Cells were then incubated with H2O2 for 1 and 6 h for the measurement of γH2AX and caspase-3 activation, respectively. To characterize the underlying mechanism, an A2AR antagonist (SCHS8261) or a PKA inhibitor (H-89) was applied after ADA treatment (as described above) for 10 or 30 min, respectively, followed by treatment with the indicated A2AR agonist. Cells were fixed and permeabilized for

Isolation of fibroblasts from patients with HD and generation of HD-iPSCs

Dermal fibroblasts from two patients (A and B) with HD were obtained through skin biopsy; written informed consent was obtained in accordance with the protocol approved by the Internal Research Board of CHANG GUNG Memorial Hospital. Four human non-HD control iPS lines (CON-iPSC-1, 2, 3 and 5) were derived from adult foreskin fibroblasts (CFB-50 iPS line) (64), newborn foreskin fibroblasts (ATCC, CRL-2429), adult dermal fibroblasts and adult peripheral blood mononuclear cells (PBMCs), respectively (Supplementary Material, Table S1). Derivation of iPSCs was performed as described previously (64).

Cultivation and neural differentiation of hiPSCs

Undifferentiated human HD-iPSCs (HD-iPSC-A1, A7, B4 and B16) and human CON-iPSCs (CON-iPSC-1, 2, 3 and 5) were routinely maintained and subcultivated as previously described (64). For neural differentiation, iPSCs were dissociated with 1 mg/ml dispase (Sigma), and then left to form embryoid bodies (EBs) in ultra-low attachment dishes (Corning) in hESC media (Dubelco’s modified Eagle’s medium (DMEM)/F12, 20% knockout serum replacement, 1% non-essential amino acids (NEAA), 2 mM L-glutamine, 100 mM 2-mercaptoethanol and 5 ng/ml basic fibroblast growth factor (bFGF)) containing 10 μM SB431542 and 100 nM LDN193189 for 3 days; EBs were then transferred to N2 media [DMEM/F12, 1X N2 supplement, 1% NEAA, 1 mM sodium pyru- vate, 2 mM L-glutamine and 20 ng/ml bFGF (all purchased from Invitrogen)] containing 10 μM SB431542 and 100 nM LDN193189 for another 2 days. Next, EBs were plated on tissue culture dishes (Nunc) in N2 media, and media were replaced every 2 days. After 3–4 weeks of differentiation, neural rosette structures (termed neural stem cells; NSCs) were manually picked and transferred to Matrigel-coated culture dishes in N2 medium. The iPS-derived NSCs were expanded as spherical aggregates.

For striatal differentiation, the iPS-derived neurospheres were picked and cut into small pieces (1–2 mm in diameter) using a needle on a weekly basis, and then plated onto new Matrigel-coated dishes in N2/B27 medium [DMEM/F12: Neurobasal medium (1:1), 0.5X N2, 0.5X B27, 1% NEAA, 2 mM L-glutamine, 10 μM SB431542 and 100 nM LDN193189] for 4 weeks; the media were subsequently replaced with B27 medium [Neurobasal medium, 1X B27, 1% NEAA, 2 mM L-glutamine] for another 4–6 weeks. At weeks 4, 8 and 12 of neural differentiation, viable cells were dissociated with TrypLE (Invitrogen, Carlsbad, CA, USA) and counted using the Trypan blue exclusion assay in a hemocytometer.

Cell stress, DNA damage and apoptosis assays

Neurons derived from iPSCs were dissociated into single cells using TrypLE, seeded onto Matrigel-coated coverslips or appropriate culture dishes and then cultured for an additional 3 days to enhance cell attachment. Cells were pre-incubated with adenosine deaminase (ADA) (1 unit/ml) for 4 h to eliminate endogenous adenosine, and then treated with an A2AR agonist (CGS21680 or APEC) of the indicated concentration for 1 h, prior to the addition of H2O2 (100 μM). Cells were then incubated with H2O2 for 1 and 6 h for the measurement of γH2AX and caspase-3 activation, respectively. To characterize the underlying mechanism, an A2AR antagonist (SCHS8261) or a PKA inhibitor (H-89) was applied after ADA treatment (as described above) for 10 or 30 min, respectively, followed by treatment with the indicated A2AR agonist. Cells were fixed and permeabilized for
immunocytochemical analysis and the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay. The TUNEL assay was performed using the DeadEnd™ Fluorometric TUNEL System (Promega, Wisconsin, USA), according to the manufacturer’s instructions. The percentage of TUNEL-positive cells was calculated from the total number of cells from three independent experiments. At least 200 cells were quantified in each case.

Genomic DNA and RNA extraction and PCR analysis of gene expression

Genomic DNA and total RNA were extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s recommendations. RNA (1 μg) was treated with DNase I (Qiagen, Germantown, MD, USA) and purified using the RNeasy kit (Qiagen, Germantown, MD, USA), before being reverse-transcribed using Superscript II (Invitrogen). The expression of total, endogenous and viral transgenes of OCT4, SOX2, KLF4 and MYC, as well as neural-related genes, were examined using semi-quantitative PCR, with previously described primers (65,66) and those in Supplementary Material, Tables S2 and S3. Quantitative PCR (RT-qPCR) was performed with SYBR Green Master and an Applied Biosystems 7900HT Fast Real-Time PCR System using gene-specific primers (Supplementary Material, Table S3).

Immunocytochemistry (ICC), immunohistochemistry and microscopy

Immunostaining was performed as previously described (29). Cells were fixed in 4% paraformaldehyde/PBS for 20 min, permeabilized with 0.05% NP-40 in PBS for 20 min and then blocked in 3% normal goat serum/PBS for 1 h. Cells were incubated with primary antibodies overnight at 4°C, washed and incubated with the corresponding secondary antibody for 60 min. Cells were counterstained with 1 μg/ml DAPI/PBS for 3 min. The primary and secondary antibodies are listed in Supplementary Material, Table S4. Images were captured on an Olympus IX71 microscope or a laser-scanning confocal microscope LSM700-meta (Carl Zeiss). The intensity of fluorescence was quantified using Image J software (National Institutes of Health, USA), and the relative intensity of fluorescence was normalized to the cell number. At least three randomly selected fields in each coverslip with a total of over 500 cells were counted for each case. For histochemical analysis, H&E staining was performed using an Autostainer XL Leica ST5010 (Leica Microsystem).

Immunoblotting

Total proteins were extracted using RIPA lysis buffer fortified with protease inhibitors (Complete Mini; Roche-Boehringer). For detection of the A2A, protein lysate was extracted using RIPA lysis buffer containing 4 M urea. Protein concentration was determined using the BCA Protein Assay Kit (Pierce). Samples (10 μl lysis buffer containing 4 M urea) were then subjected to SDS-PAGE, before being electroblotted onto a PVDF membrane (Millipore). Membranes were blocked in 1% BSA in PBS prior to sequential probing with primary antibody and HRP-conjugated secondary antibody in blocking solution. Target proteins were visualized by ECL (Amersham-Pharmacia) with an LAS-4000 mini device (FUJI Photo Film). Primary and secondary antibodies used in immunoblotting are listed in Supplementary Material, Table S4.

Knockdown of the A2A R

The A2AR knockdown constructs were produced by subcloning the 21-nucleotide shRNA sequences of the human A2AR gene (Adora2a) (Adora2a shRNA-1, 5′-AGACCTTCCGCAAGATCATTC-3′; Adora2a shRNA-2, 5′-CAGAAAGTCACCAAATCTTTT-3′) into the pLKO.1-AS101 vector. Preparation and transfection of lentiviral vectors were performed as previously described (67). CON-iPSC-derived neurons were transduced with lentiviral vectors producing shRNA targeting the A2A at a multiplicity of three in the presence of polybrene (5 μg/mL). Proteins were extracted from the transduced cells at 4 days post-transduction.

Teratoma generation

For teratoma generation, 5 × 10⁶ undifferentiated iPSCs were subcutaneously injected into NOD-SCID mice. After 10–12 weeks, mice were sacrificed and teratomas were isolated. All animal experiments were approved by the Animal Care and Use Committee of Academia Sinica, and performed in accordance with the Institutional Animal Care and Use Committee guidelines of Academia Sinica.

Microarray analysis

Total RNA was extracted from CON-iPSC-1, CON-iPSC-5, HD-iPSC-A1, HD-iPSC-A7 and HD-iPSC-B16-derived neurons at week 10 of neural differentiation. All gene expression data were obtained using the Affymetrix microarray platform by the Affymetrix Gene Expression Service Laboratory at Academia Sinica, Taiwan. Chips were scanned with an Affymetrix GeneChip Scanner 7G, and data were analyzed with GeneSpring X software (Agilent, Santa Clara, CA, USA). Raw data were normalized independently for each cell line using Robust Multichip Average. The NCBI accession number for the microarray data reported in this article is GSE59051. Changes in gene expression with a fold change >1 and an adjusted P < 0.05 were considered to be significantly different.

Statistical analyses

Unless stated otherwise, all experiments were performed in triplicate and the results are shown as the mean ± SEM. One-way analysis of variance (ANOVA) and Student’s t test were used for statistical analysis; P < 0.05 was considered significant.

Supplementary Material

Supplementary Material is available at HMG online.

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