Dopamine D2 Receptor Signaling Modulates Mutant Ataxin-1 S776 Phosphorylation and Aggregation

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Abstract

Spinocerebellar ataxia 1 (SCA1) is a dominantly inherited neurodegenerative disease associated with progressive ataxia resulting from the loss of cerebellar Purkinje cells (PCs) and neurons in the brainstem. In PCs of SCA1 transgenic (Tg) mice, the disease causing ataxin-1 protein mediates the formation of S100B containing cytoplasmic vacuoles and further self-aggregates to form intranuclear inclusions. The exact function of the ataxin-1 protein is not fully understood. However, the aggregation and neurotoxicity of the mutant ataxin-1 protein is dependent on the phosphorylation at serine 776 (S776). Although protein kinase A (PKA) has been implicated as the S776 kinase, the mechanism of PKA/ataxin-1 regulation in SCA1 is still not clear. We propose that a dopamine D2 receptor (D2R)/S100B pathway may be involved in modulating PKA activity in PCs. Using a D2R/S100B HEK stable cell line transiently transfected with GFP-ataxin-1[82Q], we demonstrate that stimulation of the D2R/S100B pathway caused a reduction in mutant ataxin-1 S776 phosphorylation and ataxin-1 aggregation. Activation of PKA by forskolin resulted in an enhanced S776 phosphorylation and increased ataxin-1 nuclear aggregation, which was suppressed by treatment with D2R agonist bromocriptine and PKA inhibitor H89. Furthermore, treating SCA1 Tg PC slice cultures with forskolin induced neurodegenerative morphological abnormalities in PC dendrites consistent with those observed *in vivo*. Taken together our data support a mechanism where PKA dependent mutant ataxin-1 phosphorylation and aggregation can be regulated by D2R/S100B signaling.

Introduction

SCA1 is one of the nine-inherited neurodegenerative diseases that belong to a group of trinucleotide repeat disorders of the nervous system. SCA1 is caused by a mutation in the ataxin-1 protein and is associated with progressive ataxia resulting from the loss of cerebellar PCs and neurons in the brainstem (Zoghbi *et al.* 2000; Koeppen 2005; Matilla *et al.* 2007). The exact function of the ataxin-1 protein has yet to be deciphered (Orr *et al.* 1993; Banfi *et al.* 1994; Matilla *et al.* 2007). Overexpression of the human mutant ataxin-1 gene in PCs of SCA1 Tg mice results in a progressiveataxia and PC pathology very similar towhat are seen in SCA1 patients (Burright *et al.* 1995). A prominent feature of PC degeneration in SCA1 Tg mice is the development of cytoplasmic vacuoles and intranuclear inclusions. The vacuolar development occurs much earlier, preceding the appearance of nuclear aggregates and the onset of behavioral abnormalities (Skinner *et al.* 2001; Vig *et al.*

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2006; Vig *et al.* 2009). The immunohistochemical analysis revealed that PC vacuoles of both SCA1 mice and human patients localize S100B protein, which otherwise is exclusively expressed in Bergmann glia (Vig *et al.* 2006; Vig *et al.* 2009). However, a definite role of glial proteins in SCA1 pathology has yet to be described.

S100B belongs to a family of Ca^{2+}-modulated proteins of the EF-hand type (Donato 1991, 1999; Zimmer *et al.* 2005). S100B is abundantly expressed in the nervous system as a cytokine with both neurotrophic and neurotoxic effects (Reeves *et al.* 1994; Huttunen *et al.* 2000; Rothermundt *et al.* 2003; Zimmer *et al.* 2005). Glial expressed S100B released from astrocytes has been shown to promote neuronal survival and development (Winningham-Major *et al.* 1989; Barger *et al.* 1995; Whitaker-Azmitia *et al.* 1997). It has been recently reported that S100B interacts with D2Rs and enhances dopamine signaling in HEK 293 cells co-expressing D2R and S100B proteins (Liu *et al.* 2008). S100B binds to the amino terminus of the third cytoplasmic loop of the D2R (Liu *et al.* 2008). Further, the D2R has a S100B-binding motif similar to TRTK12, a S100B inhibitory peptide (Ivanenkov *et al.* 1995; Bianchi *et al*. 1996; Liu *et al.* 2008). D2Rs are highly localized to cerebellar PCs and interestingly, D2Rs are down-regulated in mice lacking ataxin-1 as well as in SCA1 Tg mice (Goold *et al*. 2007). Dopamine is widely distributed in the central nervous system and is involved in the control of movement, cognition, endocrine responses and reward. Furthermore, dopaminergic abnormalities lead to many psychiatric and neurological disorders including SCA1 (Kish *et al.* 1997; Goold *et al.* 2007). D2Rs, via G-protein-coupling, inhibit adenylate cyclase and cAMP accumulation (Watts *et al*. 1996; Neve *et al.* 2004; Beaulieu *et al.* 2005; Liu *et al*. 2008).

cAMP-dependent protein kinase, PKA, is a serine/threonine protein kinase, which regulates many cellular processes including metabolism, cell cycle and cellular response to extracellular stimuli. The PKA holoenzyme consists of both regulatory and catalytic subunits. Adenylate cyclase production of cAMP increases PKA activity and the phosphorylation of PKA substrates. Therefore, factors resulting in an increase or decrease in cAMP levels are modulators of PKA activity. Here, we report that the stimulation of PKA increases both normal and mutant ataxin-1 phosphorylation and intranuclear inclusion formation in a cell culture model. Since ataxin-1 is phosphorylated by PKA at S776 (Jorgensen *et al.* 2009), the inhibition of adenylate cyclase and/or accumulation of cAMP may play a critical role in the SCA1 pathogenesis. We believe that by targeting D2R/S100B signaling, cAMP levels can be reduced, which could be therapeutically beneficial to SCA1 patients.

Materials and Methods

Materials

Bromocriptine (BRC), forskolin (FSK), H89, culture media and β tubulin antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) for cell culture was purchased from HyClone (Logan, UT, USA). Fugene 6 transfection reagent and GFP antibody were purchased from Roche (Indianapolis, IN, USA). S100B and phospho-ataxin-1 S776 antibodies were purchased from Abcam (Cambridge, MA, USA). D2R antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-calbindin D-28K (CaB) was obtained from Millipore (Temecula, CA, USA). βIII tubulin antibody was purchased from Promega (Madison, WI, USA). WT-ataxin-1 antibody was obtained from Antibodies Incorporated (Davis, CA, USA).

Cell Culture and Transfections

D2R/S100B HEK cells, previously described (Liu *et al*. 2008), were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, penicillin-streptomycin, appropriate selection antibiotics (G418 sulfate and puromycin, Sigma), and grown in an incubator at 37°C in the presence of 5% CO_2. WT HEK 293 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). GFP CMV expression vector was purchased from Clone Tech (Mountain View, CA, USA). GFP-ataxin-1[30Q] and [82Q], mammalian CMV expression vectors were kindly provided by Dr. Huda Zoghbi, Howard Hughes Medical Institute, Baylor College of Medicine (Cummings *et al*. 1998; Emamian *et al*. 2003; Chen *et al*. 2003; Vig *et al*. 2007). GFP-ataxin-1 A776 mutants were kindly provided by Dr. Harry Orr, Institute of Human Genetics, University of Minnesota (Emamian *et al*. 2003). The identities of the various GFP-ataxin-1 constructs were verified by western blotting as shown previously (Vig *et al.* 2007) and by immunofluorescence, where A776 mutants show no reaction with the phospho-ataxin-1 S776 antibodies. The GFP-ataxin-1 constructs were transformed into E. coli D5Hα cells (Invitrogen) and selected by kanamycin (Sigma) for plasmid propagation according to the manufacturer's guide lines. D2R/S100B HEK cells were transfected using Fugene 6 transfection reagent (Roche) according to the manufacture's guide lines to express the various GFP-ataxin-1 constructs.

Immunofluorescence, Western Blotting and Phosphorylation Studies

D2R/S100B HEK cells were placed on 2 well Lab-Tek chamber slides (Fisher, Houston, TX, USA) or on 6 well plates (Fisher) and transfected with either GFP vector alone, GFP-ataxin-1[30Q], [82Q] or GFP-ataxin-1[85Q] A776; cells were subjected to treatments with varying concentrations of bromocriptine (50–100nM), forskolin (30–100μM), and H89 (10–50μM) followed by immunocytochemistry as previously described (Hearst *et al*. 2009). Fixed slides were probed with the primary GFP and phospho-ataxin-1 S776 antibodies and fluorescent secondary antibodies Alexa-488 and Alexa-546 (Invitrogen, Carlsbad, CA). Cellswere observed on an Olympus BX60 epifluorescence microscope. Scoring of GFP-ataxin-1 transfected cells was performed according to the method described by Parfitt and co-workers (2009), cells were scored as soluble diffuse localization within the nucleus, as small punctuate foci or as larger inclusions (Parfitt *et al.* 2009). For western blotting, lysates were generated by resuspending cells in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate and 1% NP-40, Sigma), then sonicated as previously described (Hearst *et al*. 2009). Lysates were subjected to SDS-PAGE (4–20% acrylamide gels; Bio-Rad Laboratories, Hercules, CA, USA) as described earlier (Vig *et al*. 2009). Equal amounts of proteins were loaded in each well. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad), blocked for 1hr with blocking solution (Western Breeze, Invitrogen) and incubated overnight with appropriate concentration of GFP or phospho-ataxin-1 S776 antibodies. Immunoreactive proteins were visualized by incubation (for 1 hr) in alkaline phosphatase-labeled secondary antibody followed by reaction with the luminescent substrates (Western Breeze, Invitrogen). Western blots were analyzed using Image J software, a public domain Java image processing program provided by the Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA.

SCA1 Transgenic Mice

The SCA1 Tg mice were generated by Drs. Harry Orr and Huda Zoghbi (Burright *et al*. 1995). We maintain colonies of SCA1 Tg mice in our animal facility. The heterozygous PS-82 BO5 line of mice were identified using a transgene specific polymerase chain reaction assay and were backcrossed to the parental FVB/N strain (N=10) to establish congenic line with homogeneous background strain (Vig *et al.* 2009). The B05 line expresses 30 copies of

the transgene PS-82 to produce the mutant human ataxin-1 protein in PCs (Burright *et al*. 1995). These mice develop progressive loss of PCs and cerebellar function, where heterozygous mice become visibly ataxic at 12 weeks of age and homozygotes show ataxia at 6 weeks of age. Wild-type (WT, same background) mice were obtained from Jackson Labs, Bar Harbor, ME, USA. All animal protocols were approved by Institutional Animal Care and Use Committee (IACUC) at the University of Mississippi Medical Center (UMMC, Jackson, MS, USA).

Purkinje Cell Cultures

Cerebellar PC cultures were prepared from SCA1 Tg mice and WT mice. The whole cerebella were removed from 7 day old mouse pups. Meninges were carefully removed under the dissection microscope. Isolated cerebellar tissues were rinsed and placed in petri dishes containing cold Gey's balanced salt solution (Gey's solution, Sigma) containing 5% glucose. Tissues were cut into 250 μm slices using a McIlwain tissue chopper and resuspended in cold Gey's solution containing 5% glucose. Two to three slices were grown on Millicell membrane inserts (Fisher) using 6-well culture plates containing 1 ml plating media (vol/vol: 5% 10X Basal Medium with Earle's Salt, 2.5% 10X HBSS, 25% Horse Serum (Invitrogen), 1% 100X Pen-Strep-Glutamine, 4.5% 10% D-Glucose, 0.5% 7.5% Sodium bicarbonate and 61.5% sterile water, Sigma). Plates were incubated overnight at 35.5°C with 5% CO_2 and 100% humidity. The next day, cells were flushed and treated with various concentrations of forskolin in plating media. Cultures were fixed for immunostaining using calbindin-D28k antibody or lysed in RIPA buffer for western blotting as described above.

Tissue Processing

SCA1 Tg mice at 5 weeks of age were anesthetized and perfused with 4% buffered paraformaldehyde according to the method described by Neuroscience Associates, Knoxville, TN, USA. The brains were removed and immersed in the perfusion fixative then transferred to phosphate-buffered and processed for vibratome sectioning or paraffin embedding as previously described (Vig *et al*. 2009). Sections were cut from the midline sagittal plane of the cerebella. Sections were immunostained with D2R and S100B antibodies using the immunohistochemical protocol as previously described (Vig *et al.* 2009). Western blot analysis of the 4 and 6 week old SCA1 Tg mice and WT mice cerebellar samples were performed according to the protocol as previously described (Vig *et al.* 2009). The western blot membranes were probed overnight with the appropriate concentration of D2R and βIII Tubulin antibodies followed by alkaline phosphatase-labeled secondary antibody and luminescent substrate. The blots were visualized on hyperfilm-ECL (Amersham Biosciences, Buckinghamshire, UK).

Results

Stimulating D2R/S100B Signaling and Inhibiting PKA Activity Reduces Ataxin-1 S776 Phosphorylation and Aggregation

Dopamine D2 receptor signaling coupled to G-inhibitory-proteins is a well-established mechanism of regulating adenylate cyclase activity. Activation of adenylate cyclase generates second messenger cAMP, which increases PKA activity and phosphorylation of PKA substrates. Previously, Liu and co-workers reported that stimulation of D2R/S100B signaling by D2R agonists reduces cAMP levels in a D2R/S100B HEK stable cell line (Liu *et al*. 2008). Since phosphorylation is a common mechanism known to regulate the assembly of self-associating nuclear proteins (Emamian *et al.* 2003; Grimmler *et al*. 2005; Bansal *et al.* 2009; Hearst *et al.* 2009), we explored the impact of D2R signaling on mutant ataxin-1 phosphorylation and self-aggregation by transfecting GFP-ataxin-1[82Q] into a D2R/S100B

HEK stable cell line (Fig. 1 and 2A). D2/S100B HEK cells expressing GFP-ataxin-1[82Q], under various treatment conditions, were scored as soluble ataxin-1, small ataxin-1 foci or large ataxin-1 inclusions, as described previously by Parfitt and co-workers (Parfitt *et al.* 2009)(Fig. 3A). FSK is a well established cAMP stimulator that activates adenylate cyclase increasing PKA activity and phosphorylation of PKA substrates (Seamon *et al.* 1981; Battaglia *et al.* 1986; Chijiwa *et al.* 1990; Fujihara *et al.* 1993; Muroi *et al.* 1993; Wroblewska *et al.* 1993; Insel *et al.* 2003). Immunofluorescence of D2/S100B HEK cells expressing GFP-ataxin-1[82Q] showed a significant increase in nuclear inclusion formation after treatment with FSK (Fig. 2A and 3B). FSK induced large GFP-ataxin-1 [82Q] inclusions containing phosphorylated S776 ataxin-1 (Fig. 2A). Whereas, control cells expressing GFP-ataxin-1[82Q] showed diffuse soluble nuclear staining (Fig. 2A). To investigate the impact of D2R/S100B signaling on inclusion formation, we used BRC, a selective D2 agonist, previously shown to reduce cAMP levels in HEK cells expressing D2Rs (Watts *et al.* 1996). To explore the role of PKA in ataxin-1 inclusion formation, we used H89, a potent and selective PKA inhibitor (Chijiwa *et al.* 1990; Fujihara *et al.* 1993; Muroi *et al.* 1993; Lortet *et al.* 1999; Mao *et al.* 2007). We found that FSK induced inclusion formation was significantly reduced by 2hr pretreatment with BRC or H89 (Fig. 2A and 3B). EC_{50} for BRC, FSK and H89 were 50nM, 30μM and 5μM respectively. D2/ S100B HEK cells expressing GFP-ataxin-1[82Q] treated with H89 showed reduced phospho-ataxin-1 S776 staining compared to control. In contrast, GFP-ataxin-1[85Q] A776 transfected cells showed no phospho-ataxin-1 S776 immunostaining and failed to form inclusions (Fig. 2A). As a control, we expressed GFP-ataxin-1[82Q] and [30Q] in WT HEK cells, not expressing D2R or S100B, and treated these cells under the previously described conditions (Fig. 2B and C). We found that FSK also stimulated both GFP-ataxin-1[82Q] and [30Q] inclusion formation in WT HEK cells, whereas BRC was not effective in reducing inclusions (Fig. 3C and D). Further, H89 significantly reduced FSK stimulated inclusion formation in both GFP-ataxin-1[82Q] and [30Q] expressing cells (Fig. 3C and D). To explore the importance of ataxin-1 S776 phosphorylation site in inclusion formation, we expressed GFP-ataxin-1[85Q] A776 in WT HEK cells and treated with FSK. FSK had no effect on the inclusion formation in cells expressing A776 mutant ataxin-1 protein (data not shown). Further, for semi-quantification D2/S100B HEK cells expressing GFP-ataxin-1[82Q] treated with FSK, BRC, BRC + FSK, H89 or H89 + FSK were subjected to western blot analysis (Fig. 4A and B). FSK stimulation of PKA activity resulted in a large increase in ataxin-1 S776 phosphorylation compared to control (Fig. 4C). Furthermore, activation of D2R by BRC or inhibition of PKA by H89 greatly reduced FSK stimulated ataxin-1 S776 phosphorylation (Fig. 4C). To ensure that the various drugs were not influencing the CMV promoter activity of the various GFP-ataxin-1 constructs, we expressed GFP-ataxin-1[30Q], [82Q] and the GFP vector alone in WT HEK cells followed by the 2hr drug treatments. Western blot analysis displayed no remarkable change in the soluble GFP-ataxin-1[30Q] (Fig. 4D), GFP-ataxin-1[82Q] (Fig. 4E) or GFP (Fig. 4F) protein levels with respect to β tubulin. Overall, the cell culture data suggest that both mutant and nonpathogenic ataxin-1 S776 phosphorylation and aggregation is PKA dependent and mutant ataxin-1 phosphorylation and aggregation can be modulated through D2R/S100B signaling.

Forskolin Induces Morphological Changes in SCA1 Purkinje Cell Cultures

To further evaluate the role of PKA in the SCA1 pathology, we cultured cerebellar slices from 7 day old SCA1 Tg and WT mouse pups. PCs were identified based on CaB localization and failure to express glial fibrillary acidic protein (Vig *et al.* 2009). The slice cultures were treated with FSK followed by CaB immunostaining. FSK induced neurodegenerative morphological changes in SCA1 PCs as demonstrated by a decrease in dendritic arborizations consistent with those seen *in vivo* (Fig. 5A and B; Vig *et al.* 2009).

We saw no dendritic atrophy in WT PCs treated with FSK (Fig. 5C and D). Furthermore, to confirm our immunofluorescent data, we measured changes in PC dendritic length in both SCA1 and WT slice cultures treated with FSK compared to the controls (Fig. 5E). In SCA1 PCs, FSK significantly decreased dendritic length, while FSK had no effect on dendritic length of WT PCs. The FSK sensitivity seen in the SCA1 PC cultures further signifies that the up-regulation of PKA activity may be contributing to SCA1 PC degeneration as seen *in vivo*.

D2R/S100B Vacuoles in SCA1 Purkinje Cells

Cytoplasmic S100B vacuolar development in PCs is the earliest morphologic change seen in SCA1 Tg mice, which precedes ataxin-1 inclusion formation (Skinner *et al*. 2001; Vig *et al*. 2006; Vig *et al*. 2009). Recently, we found that S100B containing PC vacuoles also localized autophagic marker protein, microtubule-associated protein light chain 3 (LC3) (Vig *et al*. 2009). In addition, LC3-I and LC3-II, ratios were significantly altered in SCA1 mice, indicating an upregulation of autophagic processes (Vig *et al*. 2009). Autophagic clearance of toxic proteins has also been demonstrated in Huntington's and Alzheimer's diseases (Iwata *et al.* 2005; Lee 2009; Rami 2009). Mutant ataxin-1 degradation may follow a similar process, as cytoplasmic clearance of mutant ataxin-1 has been shown to involve interaction with Atg proteins (Iwata *et al.* 2005). Interestingly, we found that the cerebellar sections prepared from 5 week old SCA1 Tg mice displayed co-localization of D2R to S100B positive autophagic PC vacuoles (Fig. 6A). In Figure 6A, triangles point to S100B positive Bergmann glia and arrows point to S100B and D2R positive PC vacuoles. D2R co-localization to S100B vacuoles indicates that autophagic processes could be associated with the loss of D2Rs in SCA1 PCs. In addition, Goold and co-workers reported a decrease in D2R protein levels at 5 weeks in SCA1 Tg mice (Goold *et al.* 2007). We observed almost complete loss of D2R protein levels by 6 weeks of age (Fig. 6C and D), yet D2R protein expression remained similar to WT animals up to 4 weeks of age (Fig. 6B), suggesting that this age could be ideal for therapeutic intervention.

Discussion

Our data supports the argument that phosphorylation of mutant ataxin-1 S776 is critical for ataxin-1 mediated toxicity (Emamian *et al.* 2003; Chen *et al*. 2003; Jorgensen *et al*. 2009). In cell culture models, ataxin-1 S776 phosphorylation is coupled to inclusion formation, whereas mutant A776 ataxin-1 protein fails to form inclusions (Emamian *et al.* 2003; Chen *et al*. 2003; Jorgensen *et al*. 2009). Further evidence comes from the fact that SCA1 A776 Tg mice show lesser pathology (Chen *et al*. 2003). We demonstrate the first mutant ataxin-1 S776 phosphorylation inhibitory mechanism, where mutant ataxin-1 S776 phosphorylation and aggregation can be reduced by D2R/S100B signaling or by PKA inhibition (Fig. 3, 4 and 8). The fact that the PKA phosphorylation site motif at S776 is highly conserved implies that ataxin-1 is a PKA substrate and that ataxin-1's phosphorylation status may directly be dependent on PKA activity (Glass *et al.* 1986; Orr *et al.* 1993; Banfi *et al.* 1996; Gossen *et al.* 1996; Chen *et al*. 2003) (Fig. 7). Interestingly, in our cell culture model, even 30Q ataxin-1 formed FSK induced nuclear inclusions. This has been reported earlier in cultured HEK cells (Tsai *et al.* 2004) and is probably due to the high levels of the epsilon isoform of 14-3-3, which favors inclusions formation in cell culture, verses the predominant zeta isoform of 14-3-3 found in PCs (Umahara and Uchihara 2010). We postulate that PKA inhibitory pathways, such as D2R/S100B signaling, may impact S776 phosphorylation of both normal and mutant ataxin-1 and may be targeted to reduce ataxin-1 neurotoxicity in SCA1 (Fig. 8).

The importance of D2Rs in motor function and coordination has been demonstrated in D2R knockout mice, which have impaired locomotion and coordinated movements (Baik *et al*.

1995; Aoyama *et al.* 2000; Wang *et al.* 2000). Recent reports suggest that ataxin-1 may be an essential component of D2R gene expression in PCs of the cerebellum. Furthermore, ataxin-1 has been depicted as a transcriptional co-regulator, which alters the expression of many genes including D2R in both SCA1 Tg mice and Atxn1-null mice (Goold *et al.* 2007). SCA1 Tg mice start losing D2Rs in PCs at around 4–6 wks postnatally, which corresponds to the onset of behavioral abnormalities (Vig *et al.* 2006; Goold *et al.* 2007; Fig. 6). The loss of D2Rs may be a major contributing factor causing an upregulation of adenylate cyclase-cAMP-PKA pathways increasing neurotoxic mutant ataxin-1 S776 phosphorylation. We recently showed that S100B is internalized in cultured PCs (Vig *et al.* 2009). Since S100B complexes with D2R to inhibit adenylate cyclase and control cAMP levels (Liu *et al.* 2008), we speculate that under physiologic conditions, S100B inside PCs interacts with D2R, enhancing the regulation of PKA activity.

We have recently demonstrated that the cytoplasmic vacuoles in SCA1 PCs contain S100B protein and are formed only when mutant ataxin-1 is expressed in both SCA1 Tg mice as well as in SCA1 patients (Vig *et al.* 2009). In addition, the process of vacuolar formation may be associated with autophagy (Vig *et al.* 2009). Considering that our data demonstrates D2Rs co-localized with S100B in PC vacuoles (Fig. 6A), the continuous expression of mutant ataxin-1 to form vacuoles may be depleting functional D2Rs from the PC soma, dysregulating cAMP production and increasing PKA activity. Further evidence implicating D2Rs in the SCA1 pathogenesis comes from the beneficial effects of lithium therapy to SCA1 Tg mice (Watase *et al.* 2007). The stimulatory effects of lithium on the transcription of the D2R gene was shown in rats fed chow containing 0.2% $LiCO_3$, which resulted in an increased D2R mRNA expression level and an increased transcription rate of the D2R gene in the rat brain striatum (Kameda *et al.* 2001). It is tempting to speculate that the beneficial effects of lithium in SCA1 Tg mice could be as a result of increased D2R mRNA expression in PCs, leading to down-regulation of PKA activity.

Dopamine receptors localize not only to PC, but also to many different neurons in other regions of the brain. Dopamine distortions have been reported in SCA1, where dopamine (DA) levels were greatly reduced (−76%) in the putamen of seven patients with SCA1 (Kish *et al.* 1997). Kish and coworkers (Kish *et al.* 1997) further concluded that the degeneration in nigrostriatal DA neurons begins at the nerve ending in SCA1. Parkinson's disease (PD) like symptoms have been observed in SCA patients and recent report s by Socal and co-workers (Socal *et al.* 2009) indicated a phenotypic association between PD and CAG expansions among SCA2 and SCA3 patients. Interestingly, several SCA patients with Parkinsonian symptoms have responded well to levodopa and dopamine agonists (Geschwind *et al.* 1997; Schöls *et al.* 2004; Manto 2005). D2R agonist BRC has been used to treat PD and may be potentially therapeutic for SCA1 patients (Calne *et al.* 1974; van Hilten *et al.* 2000). Therefore, we postulate that down-regulation of the D2R pathway may enhance ataxin-1 toxicity in PCs and/or in other susceptible regions of the brain in SCA1. Taken together our data suggests that D2R agonists could be used to modulate cAMP levels and reduce PKA induced neurotoxicity in SCA1.

Acknowledgments

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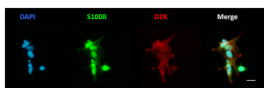Zoghbi HY, Orr HT. Glutamine repeats and neurodegeneration. Annu Rev Neurosci. 2000; 23:217–247. [PubMed: 10845064]

Figure 1.
The D2R/S100B HEK stable cell line showing co-expression of S100B and D2R proteins. Cells were immunostainfed as described in the methods section. Shown are: DAPI nuclear stain in blue, S100B in green, and D2R in red. Scale bar: 25μm.
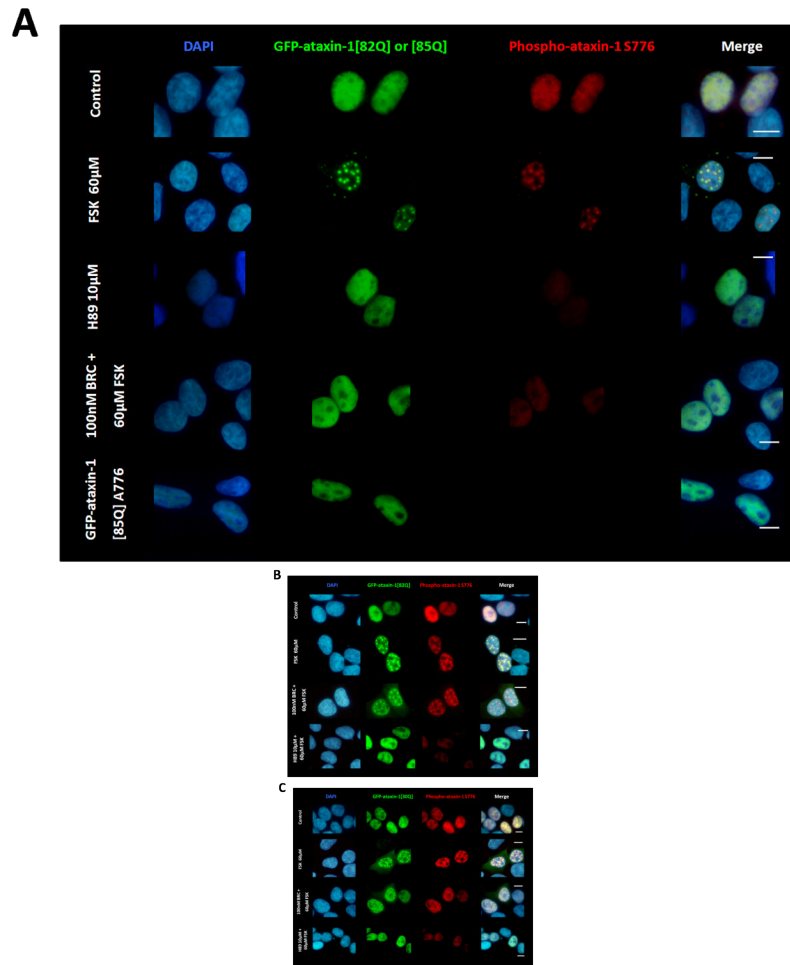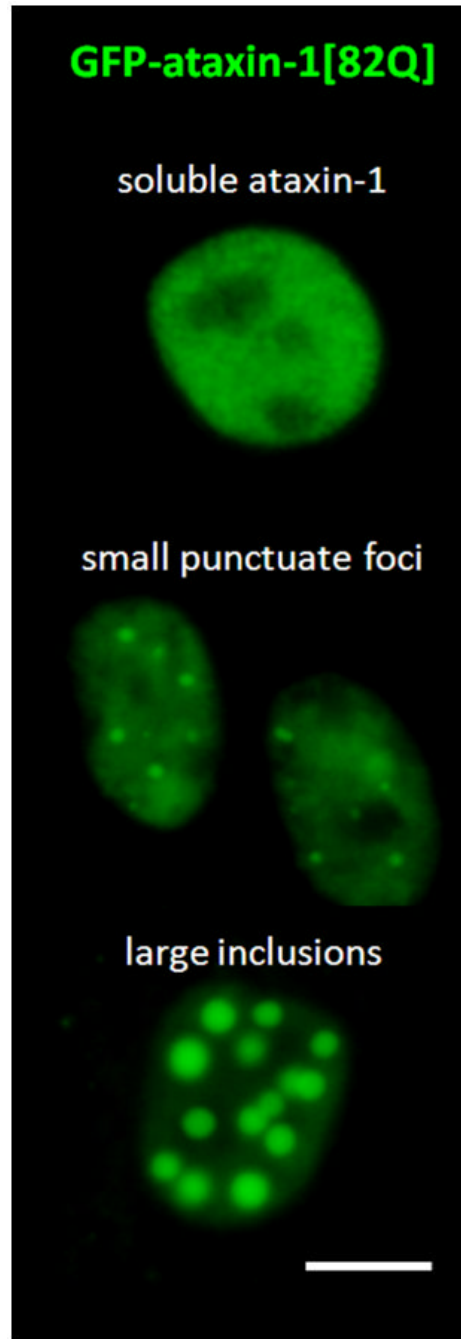
Figure 2.
(A) D2R/S100B HEK cells were transfected with GFP-ataxin-1[82Q] S776 or GFP-ataxin-1[85Q] A776. Cells expressing GFP-ataxin-1[82Q] S776 were serum starved overnight in 2% FBS, then pretreated for 2hrs with 2% FBS as the control, BRC, or H89, then treated for additional 2hrs with FSK, BRC+FSK, BRC, or control in 2% FBS. Cells expressing GFP-ataxin-1 [85Q] A776 were treated for 4hr with 2% FBS. Cells show DAPI staining in blue, GFP in green and phospho-ataxin-1 S776 in red. H89 treated cells showed less phospho-ataxin-1 S776 fluorescence. GFP-ataxin-1[85Q] A776 showed no phospho-ataxin-1 S776 fluorescence and failed to from inclusions. WT HEK cells were transfected with GFP-ataxin-1[82Q] S776 (B) and GFP-ataxin-1[30Q] S776 (C) and treated under the former conditions. BRC treatment had no impact on FSK induced inclusion formation in both GFP-ataxin-1[82Q] and [30Q] expressing cells. Scale bars: 10μm.
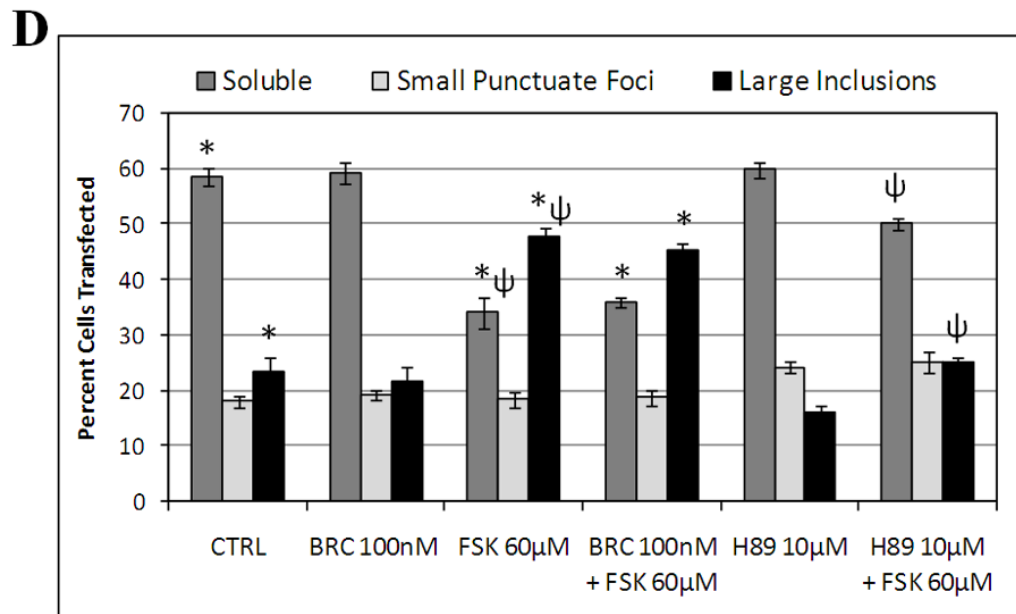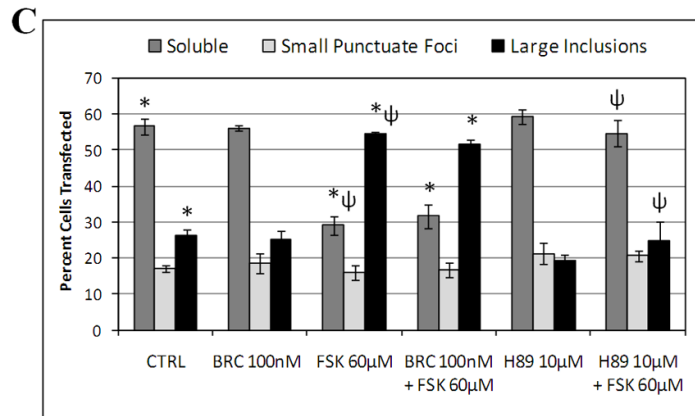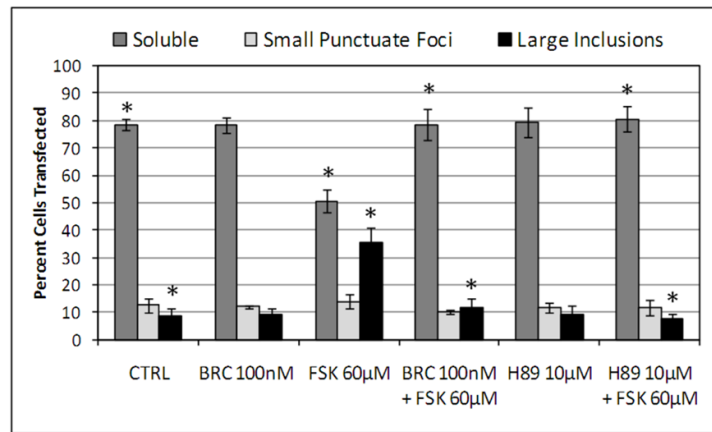
Figure 3.
(A and B) D2R/S100B HEK cells transfected with GFP-ataxin-1[82Q] were scored as soluble ataxin-1, small ataxin-1 foci, or large ataxin-1 inclusions, as previously described by Parfitt and co-workers in 2009, under the previously described treatments (Fig. 2A). Data is represented as Mean ± SE. Statistics were calculated using Student's t-test. Ctrl vs BRC,

H89, BRC+FSK or H89+FSK, P>0.05. *FSK vs CRTL, BRC+FSK or H89+FSK, P<0.005. Scale bar: 10μm. (C) WT HEK cells transfected with GFP-ataxin-1[82Q] were scored as soluble ataxin-1, small ataxin-1 foci, or large ataxin-1 inclusions, under the various described treatments. FSK vs BRC+FSK, P>0.1. *Ctrl vs FSK or BRC+FSK, P<0.01. ψFSK vs H89+FSK, P<0.01. (D) WT HEK cells transfected with GFP-ataxin-1[30Q] were scored as soluble ataxin-1, small ataxin-1 foci, or large ataxin-1 inclusions, under the various described treatments. FSK vs BRC+FSK, P>0.1. *Ctrl vs FSK or BRC+FSK, P<0.01. ψFSK vs H89+FSK, P<0.01.
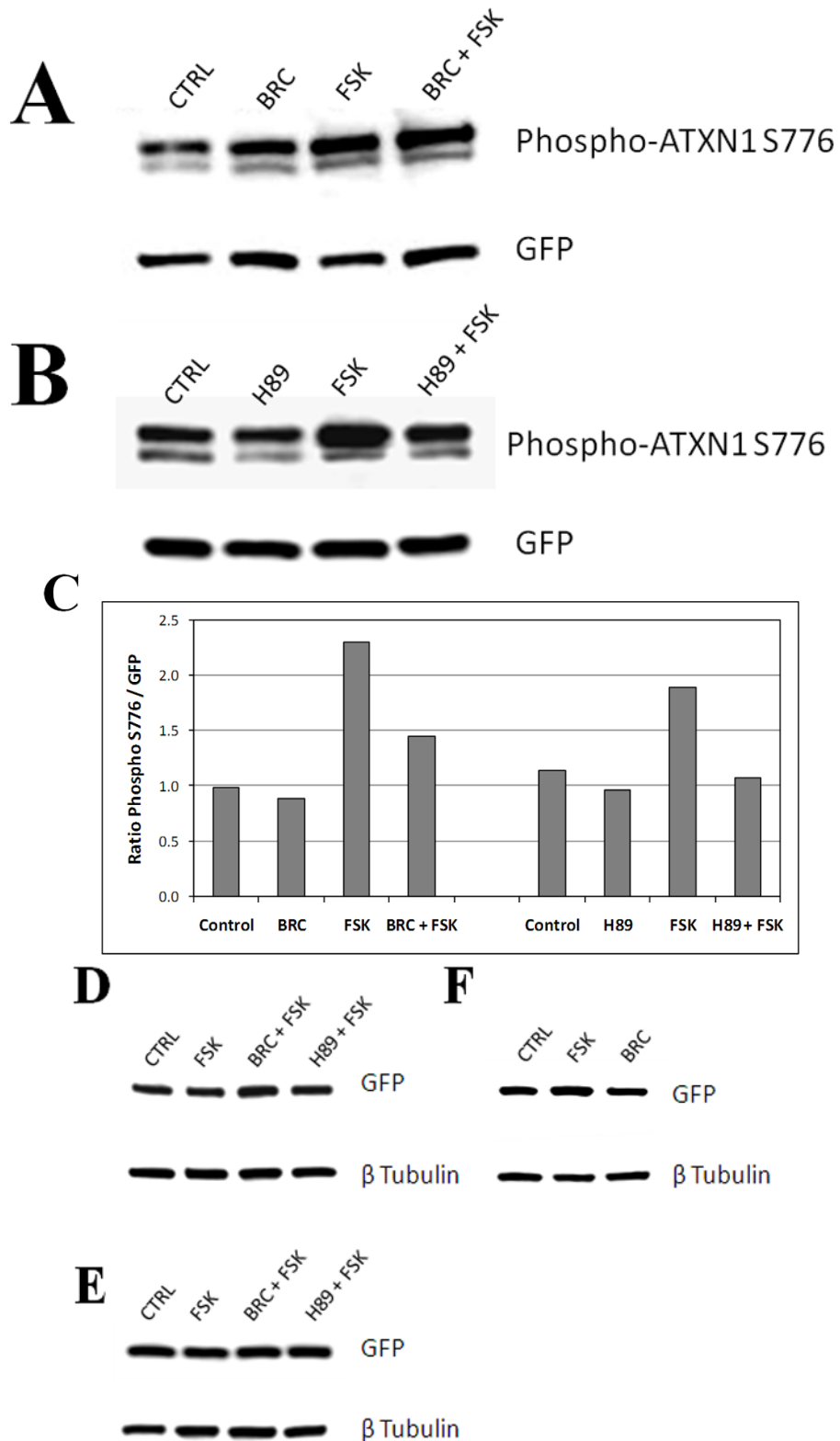
Figure 4.

Western blot of lysates from D2/S100B HEK cells expressing GFP-ataxin-1[82Q] treated as previously described (Fig. 3B) and detected with GFP and phospho-ataxin-1 S776 antibodies. (A) Lanes: Control, 100nM BRC, 60μM FSK, BRC + FSK. (B) Lanes: Control, 10μM H89, 60μM FSK, H89 + FSK. (C) Optical densities of the protein bands were taken using Image J software. FSK increased ataxin-1 S776 phosphorylation compared to control, while BRC and H89 impeded FSK stimulated S776 phosphorylation of ataxin-1. Western blots from the lysates of WT HEK cells expressing GFP-ataxin-1[30Q] (D), [82Q] (E) and GFP alone (F) were used to monitor the effect of drug treatments on the CMV promoter activity. Blots were probed with GFP and β Tubulin antibodies.
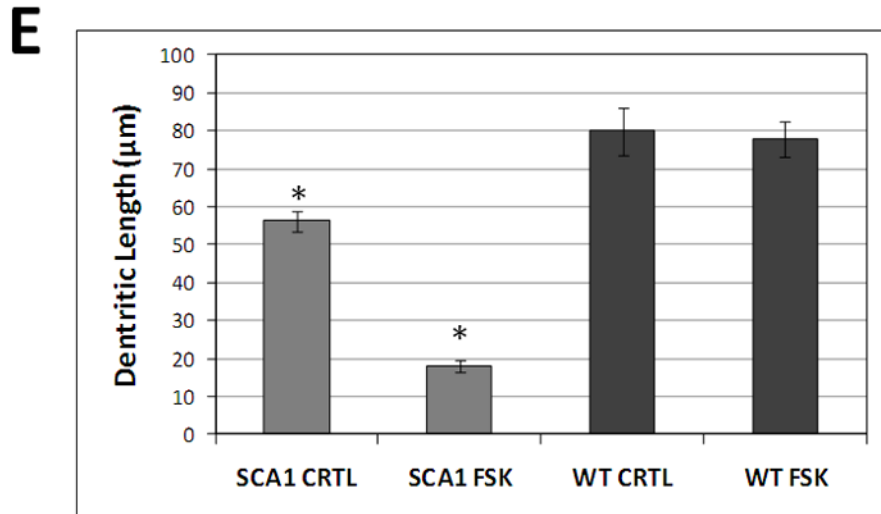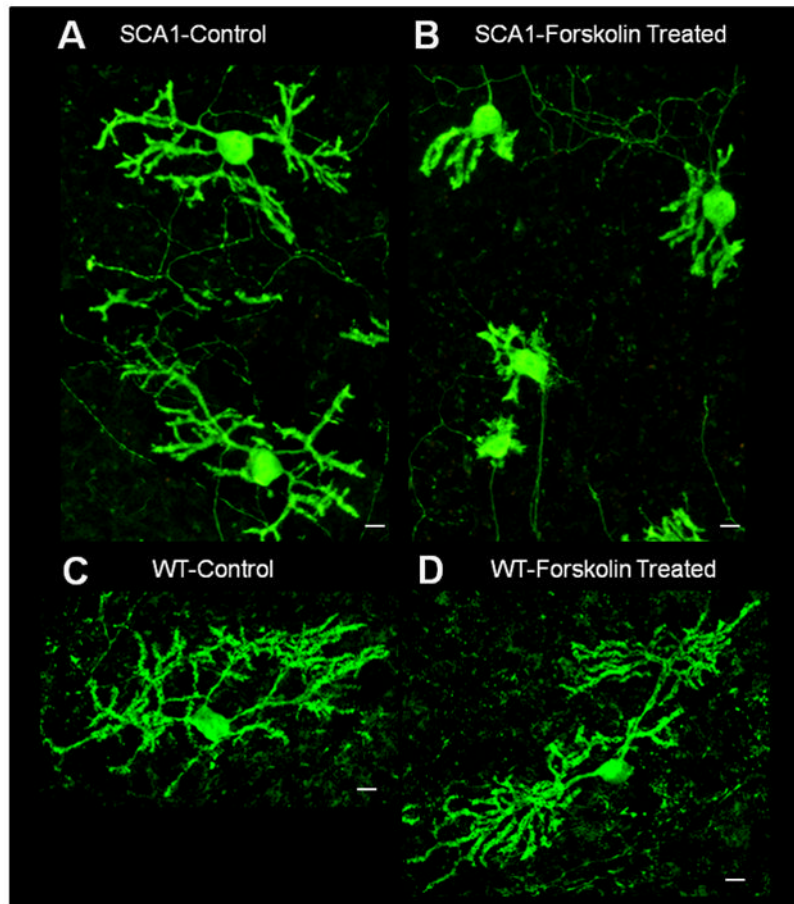
Figure 5.
Calbindin-D28k immunofluorescence of organotypic cerebellar slice cultures prepared from 7 day old SCA1 Tg mice (A and B) and WT mice (C and D). 250 μm thick slices were grown on Millicell membrane inserts using 6-well culture plates in 25% horse serum. One day old *in vitro* slices were incubated with or without 10μM FSK for 16hrs. These slices were grown for additional 5 days in the growth media followed by fixation and immunocytochemistry. The digitized image show abnormally developed dendritic processes

in 10μM FSK treated SCA1 slices (B) as compared with SCA1 control slices (A). We saw no changes in dendritic processes in 10μM FSK treated WT slices (D) as compared to WT control slices (C). Scale bar: 10μm. (E) Changes in PC dendritic length of SCA1 and WT slice cultures treated with FSK, from above digitized images, were semi-quantified using Image J software. Measurements were taken along the dendrite, from the PC soma to distal dendritic end, (n=20). Data is represented as Mean ± SE. Statistics were calculated using Student's t-test. *SCA1 slices cultures treated with FSK displayed a significant decrease in dendritic length compared to SCA1 Controls, P<0.001. WT slices cultures showed no significant changes in dendritic length with FSK treatment compared to Controls, P>0.05.
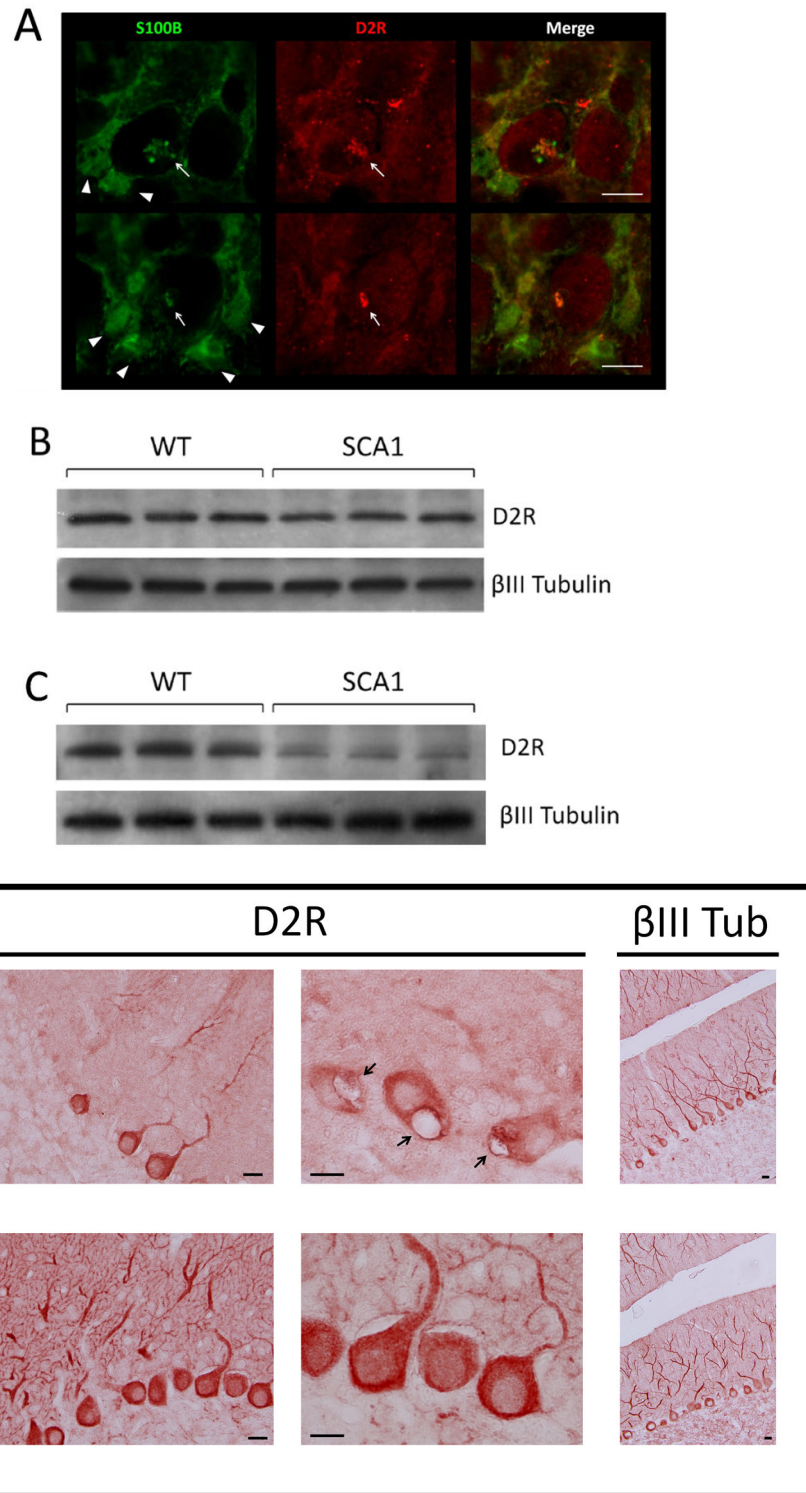
Figure 6.
(A) 5 week old SCA1 Tg mice were perfused as previously described (Vig *et al.* 2009) and 50 μm vibratome sections were immunostained for D2R and S100B. S100B immunostaining is shown in green and D2R in red. Triangles point to S100B positive Bergmann Glia and arrows point to S100B and D2R positive PC vacuoles. Scale bar: 10μm. (B) 4 week old and

(C) 6 week old SCA1transgenic mice and WT mice cerebellar extracts were subjected to western blotting loading 25μg per well followed by detection with D2R and βIII Tubulin antibodies. (D) 6 week old SCA1 Tg mice and WT mice cerebellar sections were immunostained with D2R and βIII Tubulin antibodies followed by HRP secondary staining. D2R protein levels are visibility reduced in SCA1 PCs compared to WT, although the number of βIII Tubulin positive PCs remains constant. Arrows point to SCA1 PC vacuoles. Scale bar: 10μm.

RR X S PKA phosphorylation site motif

RRWS Ataxin-1 S776

human PAATRK**RRWS**APESR
rat PTATRK**RRWS**APETR
mouse PTATRK**RRWS**APETR
xenopus PLATRK**RRWS**APETR

Figure 7.
Ataxin-1 homologs from different species have a highly conserved PKA phosphorylation site motif at S776 according to PKA motif established by Glass and coworkers in 1986 (Glass *et al.* 1986; Orr *et al.* 1993; Banfi *et al.* 1996; Gossen *et al.* 1996; Chen *et al*. 2003).
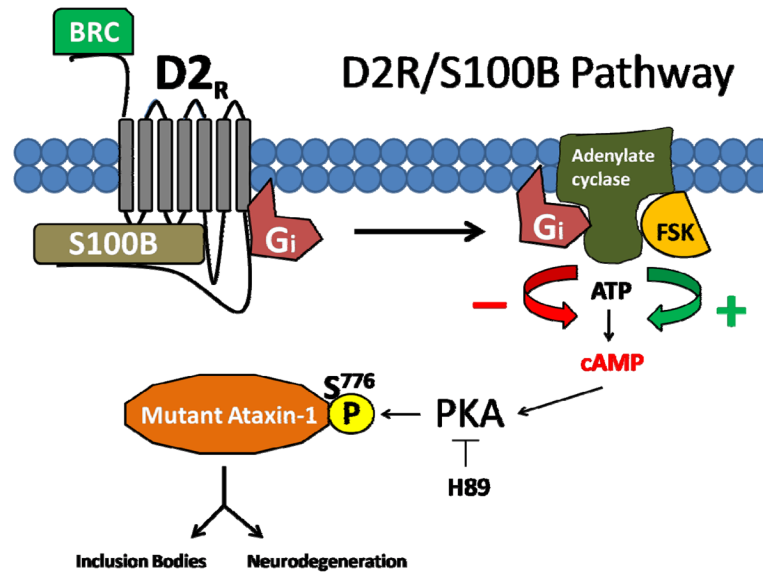
Figure 8.
D2R/S100B Signaling Pathway: D2R agonist or BRC activate G-protein-coupled inhibitory receptor, D2R, causing Gi proteins to bind and inhibit adenylate cyclase cAMP production, lowering PKA activity and decreasing S776 phosphorylation of PKA substrate ataxin-1, reducing inclusion bodies and neurodegeneration. FSK or other adenylate cyclase activator increases cAMP production and PKA activity, increasing S776 phosphorylation of PKA substrate ataxin-1, producing inclusion bodies and increasing neurodegeneration. H89 inhibits PKA S776 phosphorylation of ataxin-1. BRC could be potentially therapeutic by reducing mutant ataxin-1 S776 phosphorylation and reducing SCA1 neurodegeneration.