Glycogen Synthase Kinase-3β Inhibitors Prevent Cellular Polyglutamine Toxicity Caused by the Huntington’s Disease Mutation*

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Huntington’s disease is one of nine known neurodegenerative disorders caused by an expanded polyglutamine (poly(Q)) tract in the disease protein. These diseases are associated with intraneuronal protein aggregates. Heat-inducible chaperones like HSP70 and HSP27 suppress poly(Q) aggregation and/or toxicity/cell death. Heat shock transcription factors, including HSF-1, regulate HSP70 and HSP27 expression. HSF-1 activity is reduced by glycogen synthase kinase-3 (GSK-3) and enhanced by GSK-3 inhibitors, like lithium. Thus, we hypothesized that lithium treatment may partially rescue death in Huntington’s disease cell models. LiCl reduced poly(Q) toxicity in neuronal and nonneuronal cell lines, but this was not associated with elevation of HSP70 or HSP27. The protective effect of lithium involved GSK-3β inhibition, since poly(Q) toxicity was also reduced by SB216763, a GSK-3β inhibitor, and by overexpression of a dominant-negative GSK-3β mutant. LiCl and SB216763 increased β-catenin-dependent T-cell factor-mediated transcription. Since β-catenin overexpression protected cells from poly(Q) toxicity, we tested whether this pathway was impaired by a poly(Q) expansion mutation. Cells expressing expanded repeats had reduced β-catenin levels associated with a parallel decrease in T-cell factor-mediated transcription, compared with cells expressing wild type constructs. Since LiCl can protect against polyglutamine toxicity in cell lines, it is an excellent candidate for further in vivo therapeutic trials.

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1 The abbreviations used are: HD, Huntington’s disease; HSP, heat shock protein; GSK, glycogen synthase kinase; Tcf, T-cell factor; EGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline.
2 Huntington’s disease (HD) is a member of a family of neurodegenerative disorders caused by an abnormal CAG expansion in the coding region of the affected gene. Other members of this group include spinocerebellar ataxias 1, 2, 3, 6, and 7, dentatorubral-pallidoluysian atrophy, and spinobulbar muscular atrophy. The CAG repeats code for an expanded polyglutamine (poly(Q)) tract that causes disease by conferring a novel gain of function on the mutant protein (1). HD is characterized by expansions of a poly(Q) stretch in exon 1 of the Huntingtin gene to more than 37 glutamines, and a short N-terminal fragment encoding the poly(Q) stretch is sufficient to cause aggregates and toxicity in mice (2, 3) and in cell models (e.g. see Refs. 4 and 5). Indeed, many believe that the mutant protein acquires its toxicity and its propensity to aggregate after cleavage, forming a short (so far, incompletely defined) N-terminal fragment containing the polyglutamine stretch (5).

A pathological hallmark of HD is the formation of intracellular aggregates in specific parts of the brain in affected individuals. The exact role of these aggregates in the pathogenesis of HD is unclear; some studies have suggested that the formation of intracellular aggregates might be directly pathogenic (6–9), but others have argued that aggregates may not be deleterious (10–12). Although it is controversial whether the intraneuronal aggregates are directly pathogenic, it is more widely accepted that polyglutamine diseases are caused by misfolded, aggregate-prone proteins (13).

Cells control the levels of aggregate-prone proteins by a number of mechanisms, including molecular chaperones of the HSP40/HSP70 families. Indeed, we and others have shown that overexpression of HSP70 and HSP40 family members reduces both aggregation and cell death in cell models of HD and related conditions (reviewed in Ref. 14). We recently found that heat shock protein 27 (HSP27) also suppressed poly(Q)-mediated cell death. In contrast to HSP40/70 chaperones, we showed that HSP27 suppressed poly(Q) death without suppressing poly(Q) aggregation; our results suggested that the poly(Q) mutation induced reactive oxygen species that directly contributed to cell death and that HSP27 acted by reducing reactive oxygen species levels (14).

The expression of certain heat shock proteins (like HSP27 and HSP70) is induced by various stressors like heat, oxidants, and heavy metals. This heat shock response is mediated by heat shock transcription factors, including heat shock transcription factor 1. Recently, Bijur et al. (15) showed that overexpression of glycogen synthase kinase-3 (GSK-3) reduced activation of heat shock transcription factor 1 and production of HSP70 in response to heat shock; these effects of GSK-3 were partially reversed by treatment with lithium, which is known to inhibit GSK-3. Thus, we hypothesized that lithium treatment may enhance heat shock protein levels and partially rescue death in our HD cell models.

GSK-3β is a highly conserved, constitutively active, serine/threonine kinase that has been implicated in many fundamental cellular functions, such as the cell cycle, gene transcription, cytoskeletal integrity, and apoptosis, as a result of its ability to phosphorylate key proteins that modulate these processes (16). GSK-3β is ubiquitously expressed, including abundant expres-
sion in the brain (17). Some of the proposed target substrates of GSK-3β include transcription factors such as heat shock transcription factor 1, c-Jun, and c-Myc; enzymes that regulate metabolism such as glycogen synthase; and microtubule-associated proteins such as tau.

The activity of GSK-3β is subject to regulation by two different types of mechanisms (16). GSK-3β activity can be regulated by phosphorylation, which can be modulated by diverse pathways including insulin signaling, growth factors, and amino acids. GSK-3β is active in its nonphosphorylated form, and site-specific phosphorylation at Ser9 inhibits its activity. The second mechanism for GSK-3β regulation is independent of phosphorylation at Ser9 and involves the highly conserved Wingless (Wnt) signaling pathway, which specifies cell fate, proliferation, and differentiation in Drosophila, Xenopus, and mammals. In the absence of a Wnt signal, unphosphorylated active GSK-3β is present in a multiprotein complex (together with axin and the adenomatous polyposis coli protein, APC) in which GSK-3β phosphorylates β-catenin, thereby targeting it for ubiquitin-mediated degradation (18).

In the absence of nuclear β-catenin, transcription factors of the T-cell factor (Tcf)/lymphoid enhancer-binding factor-1 family occupy target gene promoters in a complex with various co-repressors to mediate transcriptional repression (19). When Wnt proteins bind to the frizzled receptor on the cell surface, the dishevelled protein is activated via a signaling pathway that has yet to be fully elucidated. This inhibits GSK-3 activity by disrupting the multiprotein complex, which allows β-catenin to be free, preventing its phosphorylation and degradation (20). The free accumulated cytosolic β-catenin can translocate into the nucleus, where it enhances the transcription of Wnt target genes by binding to transcription factors of the Tcf/Lymphoid enhancer-binding factor-1 family (21, 22). The target genes whose increased transcription is responsible for enhancing resistance to apoptosis have yet to be identified.

Recent work using relatively specific GSK-3β inhibitors, such as lithium or a selective small molecule inhibitor called SB216763, has supported the hypothesis that GSK-3β is pro-apoptotic. Overexpression of GSK-3β in SH-SY5Y neuroblastoma cells potentiated apoptosis, whereas lithium and SB216763 were reported to protect SH-SY5Y cells against staurosporine or heat shock-induced apoptosis (15). Part of lithium’s antiapoptotic properties may be mediated by down-regulation of p53 and up-regulation of Bcl-2 (23).

Lithium is a safe, well established drug, which is the mainstay of treatment of unipolar and bipolar disorders and is used both in the acute phase and as prophylaxis for recurrent manic and depressive episodes. Since GSK-3β may be able to regulate poly(Q) pathogenesis, lithium was an obvious choice to test as a possible pharmacological modulator of polyglutamine toxicity, since it inhibits GSK-3β.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfection Experiments, and Fluorescence-activated Cell Sorting Analysis**—African green monkey kidney cells (COS-7) and human neuroblastoma cells (SKNSH) were grown in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 100 IU/ml penicillin/streptomycin, 2 mM l-glutamine, 1 mM sodium pyruvate, and 10% fetal bovine serum and maintained at 37°C, 5% CO2 in a humidified incubator.

For experiments, the cells were pretreated for 3 days with 2.5 or 5 mM LiCl (Sigma) or for 24 h with SB216763 (Glaxo Smith Kline) prior to transfection. Treatment was maintained throughout the experimental period after transfection.

For transfection, cells were seeded in six-well plates on coverslips and grown to 60–80% confluence for 24 h. COS-7 cells were exposed to a mixture of 10 μl of LipofectAMINE Reagent™ (Invitrogen) and 2 μg of plasmid DNA for 5 h in serum-free medium, after which full culture medium was added. For efficient transfection, SKNSH cells required LipofectAMINE PLUS Reagent™ (Invitrogen); otherwise, the protocol was the same as for COS-7 cells.

In co-transfection experiments, we used HD exon 1 constructs in pEGFP-C1 (described previously in Ref. 4) and pDNA3.1 (Invitrogen) as an empty vector control; pcDNA3.1 constructs expressing wild type β-catenin containing a missense mutation of tyrosine for serine at codon 33 (S33Y), which makes it resistant to degradation (kind gifts from Hans Clevers); and a dominant negative GSK-3 β mutant (kind gift from Geoffrey Cooper). We used a 3:1 ratio of the test construct to HD exon 1 construct DNA to ensure that all cells expressing HD exon 1 constructs also expressed the appropriate test constructs. In all such experiments, we used a total of 2 μg of DNA/3.1 in serum-free medium for 4 h. To determine whether LiCl or SB216763 affected levels of EGFP-HDpolyQ expression, COS-7 cells were pretreated with LiCl or SB216763 as described above, and cells were transfected with EFPP-HDQ23. Quantitative flow cytometry was performed using a FACSort flow cytometer (BD PharMingen). 10,000–20,000 cells/sample were examined, and the data were analyzed using WinMDI software.

**Analysis of Polyglutamine-induced Aggregation**—We analyzed 300–400 EGFP-expressing cells with similar fluorescent signals per slide in multiple randomly chosen fields, blinded to the treatment condition. The proportion of EGFP-expressing cells with one or more intracellular inclusions was used as a measure of inclusion formation (14, 24, 25).

**Analysis of Cell Viability**—To assess cell viability, we looked at nuclear morphology, and EGFP-expressing cells with fragmented or condensed nuclei were counted as dead. Nuclear fragmentation was detected by nucleus staining with 4′,6-diamidino-2-phenylindole as described previously (4). We have demonstrated that these criteria are specific for cell death, since they show a very high correlation with propidium iodide staining in live cells (14). Furthermore, these nuclear abnormalities are reversed with caspase inhibitors (4, 14, 25). Analysis was performed with the observer blinded to the identity of the slides, and all experiments reported in the figures were done in triplicate at least twice. At 48 h post-transfection, cells on coverslips were washed with 1× PBS, fixed with 4% paraformaldehyde in 1× PBS for 30 min, and mounted in antifade supplemented with 4′,6-diamidino-2-phenylindole at 3 μg/ml to allow visualization of nuclear morphology using fluorescent light microscopy.

**Statistical Analysis**—Pooled estimates for the changes in inclusion formation or cell death, resulting from perturbations assessed in multiple experiments, were calculated as odds ratios with 95% confidence intervals (percentage of cells expressing construct with inclusions in perturbation conditions/percentage of cells expressing construct without inclusions in perturbation conditions)/(percentage of cells expressing construct with inclusions in control conditions/percentage of cells expressing construct without inclusions in control conditions). Odds ratios and p values were determined by unconditional logistical regression analysis, using the general log-linear analysis option of SPSS 9 software (SPSS, Chicago). Odds ratios were considered to be the most appropriate summary statistic for reporting multiple independent replicate experiments of this type (4, 14, 25), because the percentage of cells with specific inclusions under specified conditions can vary between experiments on different days, whereas the relative change in the proportion of cells with inclusions induced by an experimental perturbation is expected to be more consistent.

**Western Blotting**—Samples were trypsinized and lysed with 1× SDS-PAGE Laemmli buffer containing protease inhibitors (Roche Molecular Biochemicals). The proteins were resolved in 12.5% denaturing gels and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences) with a semidy transfer apparatus (Bio-Rad). The membranes were probed with mouse monoclonal antibodies against HSP27 and HSP70 (Stressgen Biotechnologies), Bcl-2 and p53 (Diacon Research), a mouse monoclonal anti-β-catenin antibody (Zymed Laboratories Inc., San Francisco), and a rabbit monoclonal antibody against actin (Sigma). All primary antibodies were used in a concentration of 1:1,000 except the anti-β-catenin antibody, which was used in a concentration of 1:500. Blots were probed with sheep anti-mouse or donkey anti-rabbit horseradish peroxidase-linked antibodies (Amersham Biosciences) in a concentration of 1:2,000, and bands were detected using an enhanced chemiluminescence reagent (Amersham Biosciences) according to the manufacturer’s instructions.

**Immunocytochemistry**—COS-7 cells were grown on coverslips for 16–24 h and transfected with GFP-Q74 construct or co-transfected with GFP-Q74 and the β-catenin construct. 48 h after transfection, cells were washed and fixed with 4% paraformaldehyde in PBS for 20 min and permeabilized with 0.5% Triton X-100 in PBS for 15 min. After three washes with PBS, cells were incubated in blocking buffer (5% fetal bovine serum) for 30 min. Antibodies were mouse anti-β-catenin.
LiCl protects against poly(Q)-induced cell death and inclusion formation. COS-7 (a and c) and SK-N-SH cells (b and d) were pretreated with LiCl for 3 days prior to transfection with EGFP-Q74, and the treatment was continued during the 48 h of transfection. EGFP-positive cells were scored for inclusion formation and nuclear fragmentation 48 h after transfection. LiCl treatment consistently and significantly reduced nuclear fragmentation (a and b) and reduced inclusion formation (c and d). The results represent two independent experiments each done in triplicate. 300–400 cells were counted per slide. ***, p < 0.0001; **, p < 0.001.

SB216763 protects against cell death but increases inclusion formation in cells expressing EGFP-HDQ74. Cells were pretreated with SB216763 for 24 h prior to transfection with EGFP-Q74, and the treatment was continued during the 48 h of transfection. EGFP-positive cells were scored for inclusion formation and nuclear fragmentation 48 h after transfection. SB216763 treatment significantly reduced nuclear fragmentation in COS-7 (a) and SK-N-SH cells (b) and increased inclusion formation at the same time (c and d). Me2SO (DMSO) served as a solvent control for SB216763. The results represent two independent experiments each done in triplicate. 300–400 cells were counted per slide. ***, p < 0.0001; *, p < 0.001; NS, not significant.
were mounted in antifadent containing 4',6-diamidino-2-phenylindole. Samples were observed using a confocal microscope.

**β-Catenin/Tcf Luciferase Assay**—The pTopflash (wild type) and pFopflash (mutant) constructs (Upstate Biotechnologies, Inc., Lake Placid, NY) contain a luciferase reporter under the control of two repeats each containing three copies of the Tcf binding site (wild type) upstream of the thymidine kinase minimal promoter. pFopflash contains the mutated Tcf binding sites and was used as a negative control.

COS-7 and SK-N-SH cells were pretreated with LiCl or SB216763 as described, seeded in 24-well plates, and transfected with 750 ng of pTopflash or pFopflash using LipofectAMINE PLUS™ reagent (Invitrogen or LipofectAMINE PLUS™ reagent (Invitrogen), respectively). 300 ng of a β-galactosidase reporter plasmid under the control of a constitutively active human elongation factor 1α promoter was cotransfected in each well to control for variations in transfection efficiency. Cell lysates were harvested ~24 h after transfection, and levels of luciferase and β-galactosidase activity were determined (25). The luciferase activity from each well was normalized to β-galactosidase activity. To measure and normalize the amount of luciferase activity specifically due to the presence of Tcf binding at different doses of LiCl or SB216763, we divided the luciferase activity in cells expressing pFopflash from those expressing pTopflash. Each experiment was conducted twice in triplicate, and the results were analyzed using linear regression analysis.

Stably inducible PC12 HD-Q74 (lines 1b and 10) and PC12 HD-Q23 (lines 14 and 20) (25) were transfected as described above. Cells were left uninduced or induced with 1 μg/ml doxycycline after the initial 5-h transfection period. Cells were harvested 48 h following transfection, and reporter assays were done as described above.

**RESULTS**

**Chronic LiCl Treatment Reduces Both Polyglutamine-induced Aggregation and Cell Death**—We have previously shown that transient or stable induced expression of an EGFP-tagged HD exon 1 fragment with 74 glutamines (EGFP-HDQ74) in different cell lines is associated with the formation of intracellular aggregates in a proportion of cells. EGFP-HDQ74 causes significantly more cell death, compared with the corresponding “wild type” EGFP-HDQ23, which does not form aggregates (4, 24, 25).

We tested the effect of chronic treatment with 2.5 or 5 mM LiCl on polyglutamine aggregation and cell death in COS-7 (nonneuronal) and SK-N-SH (neuronal) cell lines (Fig. 1). These LiCl concentrations mimic the extracellular fluid levels that would be seen in vivo with doses used to treat patients with bipolar affective disorder (26). We pretreated cells with LiCl for 3 days prior to transfection and continued treatment after transfection, since a number of studies (27, 28) have suggested that acute treatment does not have a protective effect against cell death. Cells were analyzed 48 h post-transfection, when 40–50% of EGFP-positive untreated/control COS-7 cells expressing EGFP-HDQ74 had inclusions and 40–45% showed nuclear fragmentation. At this time point, 40–47% of control SK-N-SH cells expressing EGFP-HDQ74 contained inclusions, and the same proportion of cells had...
fragmented/condensed nuclei. LiCl treatment showed a consistent and significant protective effect against polyglutamine-induced cell death in both cell lines (Fig. 1, a and b). LiCl also significantly reduced aggregate formation by EGFP-HDQ74 in both cell lines (Fig. 1, c and d). Odds ratios (see “Experimental Procedures”) were considered to be the most appropriate summary statistic for multiple independent experiments of this type, because the percentage of cells with nuclear abnormalities under specified conditions varied between experiments on different days, whereas the relative change in the proportion of cells nuclear abnormalities induced by a perturbation is expected to be more constant (4, 14, 24, 25).

GSK-3β Inhibition Decreases Polyglutamine-induced Cell Death—Whereas LiCl is believed to be a relatively specific inhibitor of GSK-3β, it is likely to have other effects (29). Therefore, we tested whether the protective effect of LiCl against polyglutamine-induced cell death was mimicked by the apparently specific GSK-3β inhibitor, SB216763 (30). In both COS-7 and SK-N-SH cells, SB216763 treatment was associated with a dose-dependent reduction in polyglutamine-induced cell death compared with controls (Fig. 2, a and b). However, in contrast to LiCl, SB216763 was associated with an increase in the proportion of cells with aggregates (Fig. 2, c and d). To further confirm that GSK-3β inhibition could reduce polyglutamine-induced cell death, we used a dominant negative GSK-3β mutant (31) in a co-transfection experiment together with EGFP-HDQ74 in SK-N-SH cells. As shown in Fig. 3, the dominant negative mutant had the same effect as SB216763 and decreased polyglutamine-induced cell death but increased the proportion of cells with aggregates (compared with an empty vector control).

Compared with untreated control cells, neither LiCl nor SB216763 (at the highest concentrations used) were toxic to the cells, and neither compound modulated the low background levels of cell death in the cells expressing EGFP-HDQ23, as assessed by flow cytometry (data not shown; see “Experimental Procedures”). We examined levels of protein expression by flow cytometry using EGFP-HDQ23, since transient transfection with this construct gives uniform cytoplasmic EGFP expression and does not interfere with the fluorescence read-out signal. In contrast, a proportion of cells expressing the mutant EGFP-HDQ23 constructs form intracellular aggregates, which tend to sequester all the cytoplasmic EGFP and result in a spuriously low fluorescence read-out signal. Neither LiCl nor SB216763 (at the highest concentrations used) modulated EGFP-HDQ23 expression.

LiCl Treatment Does Not Mediate Its Effects by Up-regulating HSP27, HSP70, or Bcl-2—To test whether LiCl and SB216763 protected against polyglutamine-induced toxicity by up-regulating the levels of stress-inducible heat shock proteins, we analyzed Western blots on COS-7 and SK-N-SH cells, which had been treated with LiCl or with SB216763 according to the protocol that had been used for the EGFP-HDQ74-transfected cells. Neither compound induced any significant changes in the expression levels of HSP70 or HSP27 (Fig. 4, a and b).

Since LiCl may mediate antiapoptotic effects by up-regulating Bcl-2, we tested whether overexpression of a Bcl-2 construct together with EGFP-HDQ74 could rescue polyglu-
tamine-induced toxicity. We found that Bcl-2 overexpression significantly reduced polyglutamine-induced death in SK-N-SH cells (Fig. 5). However, this mechanism is unlikely to be responsible for the protection mediated by LiCl or SB216763 in our HD model, since neither compound induced detectable changes in the levels of protein expression of Bcl-2 or p53 (which negatively regulates Bcl-2 expression) (Fig. 4, c and d).

LiCl Activates a Prosurvival Pathway through GSK-3β Inhibition and Activation of β-Catenin/Tcf-mediated Transcription—To determine whether LiCl had an effect on GSK-3β-mediated β-catenin signaling, we used the pTopflash or pFopflash luciferase reporter plasmids. The pTopflash (wild type) and pFopflash (mutant) constructs contain a luciferase reporter under the control of two repeats, each containing three copies of the Tcf binding site (wild type) upstream of the thymidine kinase minimal promoter. pFopflash contains mutated Tcf binding sites and was used to control for the transcriptional activity of the reporter that was independent of Tcf elements. We co-transfected COS-7 cells, pretreated with LiCl or SB216763, with pTopflash or pFopflash and a β-galactosidase reporter plasmid under the control of a constitutively active human elongation factor 1α promoter in a 3:1 ratio. β-Galactosidase activity was quantified to control for transfection efficiency in each well. In each condition, we compared the wild type with mutant Tcf-containing vectors to determine the extent of transcriptional activation specifically due to the Tcf elements. LiCl treatment caused a dose-dependent increase in β-catenin-mediated transcription (p < 0.0001, r² = 0.77), and this effect was even more pronounced with SB216763 treatment (p = 0.0004, r² = 0.89) (Fig. 6).

To confirm that the protective effect of LiCl against polyglutamine-induced toxicity could be mediated through activation of Tcf transcription, we overexpressed either wild type β-catenin or the S33Y β-catenin mutant, which is resistant to degradation (32), together with EGFP-HDQ74 in COS-7 and SK-N-SH cells. As shown in Fig. 7, overexpression of either wild type β-catenin or the S33Y β-catenin mutant in COS-7 cells and SK-N-SH cells were transfected with wild type β-catenin or S33Y β-catenin mutant and EGFP-HDQ74 in a 3:1 ratio. Cells were scored for inclusion formation and nuclear fragmentation 48 h after transfection. Overexpression of both wild type β-catenin and S33Y β-catenin mutant consistently and significantly reduced nuclear fragmentation in both cell lines (a and c) and significantly increased inclusion formation in both COS-7 (b) and SKNSH cells (d). The results represent two independent experiments each done in triplicate. 300–400 cells were counted per slide. ***, p < 0.0001; **, p < 0.001.

**Fig. 8.** Tcf-mediated transcription is reduced in PC12-stable inducible cell lines expressing EGFP-HDQ74, compared with EGFP-HDQ23. Data are shown for two independent clonal lines with each repeat length (EGFP-HDQ74.1b, EGFP-HDQ74.10, EGFP-HDQ23.14, and EGFP-HDQ23.10) induced for 48 h. Specific Tcf-mediated luciferase activity was determined by comparing pTopflash activity with pFopflash activity, and the results are expressed as a ratio of pTopflash/pFopflash. Data represent means and S.E. of two experiments each in triplicate. Similar significant trends were observed if we subtracted pFopflash from pTopflash activities.

**Fig. 9.** β-Catenin levels are reduced in cells expressing expanded poly(Q) repeats. Western blot showing down-regulation of endogenous β-catenin in PC 12 cells expressing EGFP-HDQ74 (lanes 1 and 2) as compared with cells expressing EGFP-HDQ23 (lanes 3 and 4). Data are shown for two independent clonal lines for both EGFP-HDQ74 and EGFP-HDQ23, each induced for 48 h. The blot was reprobed with anti-actin antibody as a control for loading. This result was consistently reproducible. Lane 1, EGFP-HDQ74.10; lane 2, EGFP-HDQ74.1b; lane 3, EGFP-HDQ23.20; lane 4, EGFP-HDQ23.14.
and SK-N-SH cells decreased polyglutamine-mediated cell death (compared with an empty vector control) but increased the proportion of EGFP-positive cells with inclusions. The trend toward an enhanced protective effect of the S33Y mutant in SK-N-SH cells is compatible with its greater stability.

**β-Catenin Levels and Tcf-mediated Transcription Are Reduced in Cells Expressing Expanded Polyglutamine Repeats—** The above data suggest that GSK-3β inhibition rescues polyglutamine-mediated cell death by increasing levels of Tcf-mediated transcription, which is mediated by β-catenin. Thus, we tested whether this component of the Wnt signaling pathway was impaired by the HD mutation. To avoid the confounds of triple transfection and cell death in reporter gene studies of promoter activity, we studied this pathway in our PC12-stable doxycycline-inducible cell lines (25). These lines, which we have previously characterized, show minimal cell death for the first 3 days after induction (25) and allow us to analyze promoter activity using conventional double transfection with the luciferase reporter gene of interest and β-galactosidase-expressing vector to control for transfection efficiency. We studied two independent mutant and two independent wild type lines induced to express either EGFP-HDQ74 or EGFP-HDQ23 constructs. Cells were transfected with pTopFlash or pFopFlash vectors 5 h before induction and analyzed 48 h after induction. Fig 8 shows that Tcf-mediated transcription was significantly reduced in the HDQ74 lines, compared with the HD-Q23 lines. This reduction was associated with a parallel reduction in β-catenin levels (Fig. 9).

A plausible explanation for this effect was that β-catenin may be sequestered to inclusions. However, neither endogenous nor exogenously transfected β-catenin was associated with nuclear and cytoplasmic inclusions formed by EGFP-HDQ74 in COS-7 cells (data not shown). We also observed no association of GSK-3β with inclusions (data not shown).

**DISCUSSION**

Our data show that inhibition of GSK-3β by LiCl, SB216763, or a dominant negative GSK-3β construct protects against polyglutamine-induced death in SK-N-SH and COS-7 cells. These protective effects are mediated, at least in part, by β-catenin, since LiCl and SB216763 enhance the β-catenin-dependent Tcf transcription pathway and β-catenin overexpression protects against polyglutamine-induced death. The protective effect of LiCl at concentrations mimicking what would be seen at therapeutic doses in vivo suggests that this drug needs serious consideration for therapeutic trials in HD mouse models, which we are planning. However, such trials will need to be carefully conducted, given the narrow therapeutic range of this drug.

Whereas all of the compounds with GSK-3β-inhibitory activity reduced cell death caused by the HD mutation, it was interesting that LiCl reduced the proportion of cells with aggregates but SB216763, the dominant-negative GSK-3β, and β-catenin increased the proportions of cells with aggregates. It is possible that LiCl may perturb other pathways unrelated to GSK-3β (29). If GSK-3β inhibition modulated cell death but not aggregation, then the apparent dissociation between aggregation and cell death seen with SB216763, the dominant-negative GSK-3β, and β-catenin would occur if aggregates were protective, epiphenomena, or deleterious; aggregate formation will be more likely in cells that remain attached to a coverslip for longer times due to the reduced rate of cell death.

The protective effects of GSK-3β inhibition in our model did not appear to be associated with a heat shock response or due to elevated levels of Bcl-2 and decreased p53. Whereas some studies have suggested that GSK-3β modulates these pathways, Cohen and Frame (33) have argued that many of the putative GSK-3β substrates have not yet been shown to fulfill series of criteria that are needed for them to be considered as true physiological targets.

Since overexpression of β-catenin rescued polyglutamine-induced cell death, we tested whether this pathway was impaired in our HD cell model. In cells expressing expanded repeats, there was a reduction in β-catenin levels that was associated with a parallel reduction in Tcf-mediated transcription, as compared with cells expressing wild type constructs. It is unclear how the polyglutamine expansion mutation exerts this effect. Neither β-catenin nor GSK-3β was associated with aggregates. It may be difficult to elucidate how the HD mutation affects β-catenin levels, since many of the details of the Wnt signaling pathway upstream of GSK-3β have yet to be elucidated.

In conclusion, we find that GSK-3β inhibition rescues polyglutamine-induced cell death in neuronal and nonneuronal cell lines. This protection is mediated by increases in β-catenin and its associated transcriptional pathway. This pathway is likely to be relevant to the polyglutamine-induced cell death in our models, since we demonstrated decreased levels of β-catenin and Tcf-mediated transcription in cells expressing expanded repeats. Since LiCl, a commonly used drug, can protect against polyglutamine toxicity via this pathway in both neuronal and nonneuronal cell lines, it is an excellent candidate for further in vivo therapeutic trials.

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