Down-regulation of the dopamine receptor D2 in mice lacking ataxin 1

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Ataxin 1 (Atxn1) is a protein of unknown function associated with spinocerebellar ataxia type 1 (SCA1), a neurodegenerative disease of late onset with variable degrees of cerebellar ataxia, ophthalmoplegia and neuropathy. SCA1 is caused by the toxic effects triggered by an expanded polyglutamine (polyQ) within Atxn1 resulting in neurodegeneration in the cerebellum, brain stem and spinocerebellar tracts. To gain insights into Atxn1 function, we have analysed the cerebellar gene expression profiles by microarray analysis in Atxn1-null mice, and identified alterations in expression of genes regulated by Sp1-dependent transcription, including the dopamine receptor D2 (Drd2), retinoic acid/thyroid hormone and Wnt-signalling. Interestingly, Drd2 expression levels are reduced in both Atxn1-null and transgenic mice expressing a pathogenic human Atxn1 with an expanded polyglutamine in cerebellar Purkinje cells. Our co-transfection experiments in human neuroblastoma SH-SY5Y cells and luciferase assays provide evidence for transcriptional regulation of Drd2 by Atxn1 and its AXH module. We show that Atxn1 occupies at the Drd2 promoter in vivo, and interacts and functions synergistically with the zinc-finger transcription factor Sp1 to co-regulate Drd2 expression. The interaction and transcriptional effects are mediated by the AXH domain within Atxn1 and are abrogated by the expanded polyQ within Atxn1. Therefore, this study identifies novel molecular targets that are regulated by Atxn1 which might contribute to the motor deficits in SCA1, and provides new insights into the mechanisms by which Atxn1 co-regulates transcription.

INTRODUCTION

Spinocerebellar ataxia type 1 (SCA1, OMIM #164400) is a late onset autosomal dominant neurodegenerative disorder characterized by cerebellar ataxia associated with variable degrees of oculomotor abnormalities, pyramidal, extrapyramidal features, peripheral neuropathy and cognitive impairment (1,2). The basic genetic defect in SCA1 consists of the expansion of a translated trinucleotide CAG repeat located within exon 8 of the SCA1 gene (3). The CAG repeat encodes for a polyglutamine (polyQ) tract, and the disease symptoms are triggered by the expanded polyQ located within the N-terminus region of ataxin 1 (Atxn1), a 98-kDa protein of unknown function (3). Thus, together with SCA1, at least eight additional inherited neurodegenerative diseases, including Huntington’s disease (HD), Spinobulbar muscular atrophy, dentatorubral pallidoluysian atrophy (DRPLA) and SCA subtypes 2, 3, 6, 7 and 17 are known to be caused by expansions of glutamine-encoding repeats in genes whose sequences are otherwise unrelated (4). Compelling evidence indicates that common molecular pathways and biological mechanisms might underlie neurodegeneration in these diseases.

The mechanisms underlying SCA1 pathogenesis are still not completely understood, but some general principles have emerged. Genetic studies in mice and flies support a toxic gain-of-function mechanism since mice lacking Atxn1 do not develop ataxia or cerebellar Purkinje cell (PC) pathology (5). This argues against a loss-of-function or haploinsufficiency of Atxn1 as the underlying pathogenic mechanisms in SCA1. It is generally assumed that the expanded polyQ
causes mutant Atxn1 to misfold and form insoluble aggregates in the nucleus leading to neuronal dysfunction and eventually cell death in SCA1 (4). A possible mechanism for aggregate formation by mutant Atxn1 would be by loss of native state stability by the expanded polyQ and, thus, leading to the formation and accumulation of a partially unfolded, aggregation-prone protein, resulting in fibrillization. Changes in conformation may also enable mutant Atxn1 to recruit cellular proteins through aberrant interactions and alter cell-type-restricted functions. While the role of the polyQ expansion in SCA1 is well established, the contribution of the protein framework is becoming increasingly clear in the pathogenic mechanisms of neurodegeneration. In this regard, both the AXH domain, a highly conserved globular module near the C-terminus of Atxn1 exhibiting significant sequence similarity to a region in the High-mobility group box 1 (HB1P1) transcription factor (6,7), and phosphorylation of Atxn1 in Ser776 (8,9), appear to determine toxicity in flies and mice (8,10). Transcriptional dysregulation mediated by the soluble, non-aggregated mutant protein is an important feature of the pathogenic mechanisms and precedes the onset of disease symptoms in SCA1 (11,12). This might result from interactions of Atxn1 with several transcriptional co-regulators, including the leucine-rich acidic nuclear protein (LANP/ANP32A) (13), polyQ-binding protein (PQBP1) (14), silencing mediator of retinoid and thyroid hormone receptors (SMRT) (15), Boat (16), Gfi-1/Senseless (10), Capicua (17) and the nuclear hormone coactivator Tip60 (18). Overall, these observations indicate that the expanded polyQ might interfere with the cellular function of Atxn1.

While much effort has been dedicated to elucidate the molecular pathogenic mechanisms in SCA1, only few studies have addressed the biological function of Atxn1. A transient burst of mRNA expression of Atxn1 occurs at P14 when the murine cerebellar cortex becomes physiologically functional (19). This evidence together with more recent data (18) implicate a role for Atxn1 at specific stages of cerebellar development. Loss of Atxn1 function in Sca1<sup>−/−</sup> knockout (KO) mice leads to hippocampal alterations in short-term synaptic plasticity, spatial learning impairment and motor deficits in the absence of obvious anatomical changes (5).

Herein, we have analysed the cerebellar gene expression profiles in Sca1<sup>−/−</sup> mice. We hypothesized that identifying the genes and transcriptional pathways regulated by wild-type (WT) Atxn1 could reveal molecular pathways that might be altered in SCA1. We have identified altered expression of genes regulated by Sp1-dependent transcription, including the dopamine receptor D2 (Drd2), retinoic acid (RA)/thyroid hormone, and Wnt-signalling in mice lacking Atxn1. Drd2 is decreased in cerebella of null mice lacking Atxn1 and transgenic mice expressing human Atxn1 with an expanded polyQ in PC. Our co-transfection experiments in human neuroblastoma SH-SY5Y cells and luciferase assays provide evidence for transcriptional regulation of Drd2 by Atxn1 and its AXH module. We demonstrate that Atxn1 occupies at the Drd2 promoter in vivo, and interacts and functions synergistically with the zinc-finger transcription factor Sp1 to co-regulate Drd2 expression. The interaction and transcriptional effects are mediated by the AXH domain within Atxn1 and are abrogated by the expanded polyQ. Therefore, this study identifies novel molecular targets regulated by Atxn1 that might contribute to the motor deficits in SCA1 and provides new insights into the mechanisms of Atxn1 co-regulating transcription.

RESULTS

Cerebellar gene expression profiles of Atxn1-null mice

Genome-wide microarray analysis interrogating 22,690 murine genes (Affymetrix MOE430A) enabled us to identify 119 genes with a 1.5-fold change value in cerebella of 5-week-old Atxn1-null mice compared with age-matched WT mouse cerebella. Significant analysis of microarrays (SAM) (20) identified 59 (58%) positive and 43 (42%) negative significant changes with a false discovery rate (90th percentile) of 4.61% (Supplementary Material, Table S1 and Fig. S1). Hierarchical average pairwise-linkage unsupervised clustering grouped genes according to relative variation in gene expression patterns (Fig. 1). Genes showing altered expression levels in Atxn1-null mice were grouped into three general functional categories: biological function, molecular function and cellular component according to the biological process ontology of the Gene Ontology Consortium (21). Nineteen non-redundant functional annotation charts for enriched (P < 0.05) categories were generated with DAVID (22) (Supplementary Material, Table S2). Of interest are the identified genes implicated in translation initiation (P-value = 1.71 x 10^{-3}), Wnt-signalling (P-value = 1.26 x 10^{-2}), retinol binding (P-value = 2.55 x 10^{-2}), nucleic acid binding (P-value = 2.61 x 10^{-2}) and intracellular signalling cascade (P-value = 3.45 x 10^{-2}) (Table 1). Microarray data was validated by semi-quantitative real-time RT–PCR (Q-RT–PCR) to confirm differential expression in mutant samples for a subset of representative genes identified in our analysis. ANOVA revealed significant differences in mRNA levels in all 10 transcripts analysed (Fig. 2). These results uniformly confirmed representative array data and gave us confidence in the significant microarray data identified by SAM. Microarray MIAME-compliant data was deposited in the ArrayExpress database (ArrayExpress accession number: E-MEXP-886).

Our microarray analysis identified alterations in gene expression of the related zinc-finger transcription factors Sp1 and Kruppel-like factors 4 and 9, which bind GC-rich DNA elements to regulate transcription (23). We detected dysregulation of several genes with Sp1-binding sites in their promoters, such as Drd2, the dopamine (DA) neurotransmitter transporter (Dat), also known as solute carrier family 6 member 3 (Slc6a3), hippocalcin (Hpcal), neurogranin (Ngrn), platelet activating factor (Pafah1b3), thyroid hormone receptor interactor 4 (Trip4), phosphatidylinositol glycan c and x (Pigc and Pgcx) and the orphan nuclear receptor COUP-TFI (Nrf2). This data pointed to a possible role for Atxn1 in regulating Sp1- and/or Sp1-like-dependent transcription. Another subset of genes dysregulated in cerebellum of Atxn1 KO mice are regulated by RA and/or thyroid hormone, such as Drd2, Nr2f2, the cellular retinol binding protein 1 (Rbp1), trangryrin (Ttr) and Trip4 (24). These findings are of particular interest since Atxn1 has been functionally linked to the retinoid and thyroid hormone receptors signalling pathways (15,18).
Figure 1. Cerebellar gene expression profiles of 5-week-old 129/SvJ mice lacking Atxn1. Matrices visualizing hierarchical clustering of differentially expressed up-regulated (59 genes) (A) and down-regulated (43 genes) (B) significant genes detected with SAM with a false discovery rate (90th percentile) of 4.61%. Each gene is visualized as a single row of coloured squares, with one square for each sample, and each sample is represented by a single column. The colour indicates the relative expression of the gene with lighter colours noting lower levels of expression, where the colour scale ranges from log ratios of −3.0 to 3 as shown in the scale bar on the top of the figure. Genes exhibiting increases or decreases in basal activity are shown in red or green, respectively. Gene symbols are indicated in the lists on the right of the matrices. WT, wild-type mice; KO, homozygous Atxn1-null mice; (n = 5).
Retinoic binding (P-value = 1.71 × 10⁻³)
Basic leucine zipper and w2 domains 2 (Bzw2) (↑ 1.6)
Eukaryotic translation initiation factor 1a (Eif1a) (↑ 0.6)
Wnt-receptor signalling pathway (P-value = 1.26 × 10⁻²)
Cyclin-dependent kinase 2 (Cdk2) (↑ 1.6)
Sclerostin domain containing 1 (Sostdc1) (↑ 3.7)
Sine oculis-related homeobox 3 (Six3) (↑ 0.6)
Transducin-like enhancer of split 3 (Tle3) (↑ 0.6)
Wingless-related MMTV integration site 5A (Wnt5a) (↑ 1.8)

Table 1. Summary of enriched GO categories of significant genes identified by SAM

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<tr>
<th>GO Category</th>
<th>P-value</th>
<th>log2 C2</th>
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<tr>
<td>Retinal binding</td>
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<td>Amyloid beta (a4) precursor-like protein 2 (Aplp2)</td>
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<tr>
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<tr>
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<td>Homeobox A5 (Hoxa5)</td>
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<td>Kruppel-like factor 4 (Klf4)</td>
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<td>Kruppel-like factor 9 (Klf9)</td>
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<tr>
<td>Period homolog 2 (Per2)</td>
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<tr>
<td>Sine oculis-related homeobox 3 (Six3)</td>
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<td>Integrin-related cell adhesion molecule 1 (Itg1)</td>
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<td>Intracellular signalling cascade (P-value = 3.45 × 10⁻²)</td>
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<td>Neurogranin (Ngrn)</td>
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<td>Rap2c, member of ras oncogene family (Rap2c)</td>
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<td>Src-like adaptor (Sla)</td>
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<td>Unc-51-like kinase 1 (Ulk1)</td>
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<tr>
<td>Wingless-related MMTV integration site 5A (Wnt5a)</td>
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Expression images obtained from the Allen Brain Atlas (25) corresponding to Atxn1, hippocalcin (hpcA), neurogranin (Ngrn), dopamine receptor d2 (drd2) and DA neurotransmitter transporter (Dat/Slc6a3) are shown in Supplementary Material, Fig. S2.

Alterations of Drd2 levels in Atxn1-null and Sca1 transgenic mice

Drd2 is down-regulated in brains from 5-week-old Atxn1-null mice (Fig. 3A and B). KO mice have significantly decreased Drd2 levels in both cerebellum (2.12-fold reduction, F1,14 = 22.363, P < 0.001; n = 8) and the rest of the brain (1.62-fold reduction, F1,14 = 8.73, P = 0.01; n = 8). Drd2 distribution was examined in brains from WT and KO mice using immunohistochemistry with α-Drd2 monoclonal antibodies. Drd2 immunoreactivity was detected as a fine, punctate immunostain in neuronal cell bodies of spiny medium neurons of the striatum, neurons of the hippocampal CA1–CA4 areas and granule cells of the dentate gyrus, olfactory bulb, pyramidal and non-pyramidal neurons of the cerebral cortex, hypothalamic and thalamic nuclei, dopaminergic neurons in the midbrain (A8–A10 groups) and cerebellum (data not shown). In cerebellum, Drd2 immunostaining was moderately present in a proportion of PC, and intensive in Golgi cells of the granule cell layer and deep cerebellar nuclei neurons. No significant immunolabelling was observed in basket, granule and stellate cells. It was not possible to determine significant differences in staining in Atxn1-null mice when compared with age-matched control littermates due to variations in staining intensity between mice of each group.

Significant Drd2 expression alterations were also detected in cerebella from transgenic mice expressing either a non-pathogenic human SCA1 allele (A02: 30 CAG repeats) or a pathogenic human SCA1 allele (B05: 82 CAG repeats) (F2,15 = 51.95, P < 0.001; n = 6) (Fig. 3C and D) (26). It is important to note that although the detected differences in Drd2 levels in cerebellar extracts from A02 and B05 mice when compared with WT are small (A02/WT: 1.13-fold, P < 0.001; B05/WT: 0.91-fold, P = 0.029), they are caused by expression of Atxn1 only in PCs of the cerebellar cortex in A02 and B05 mice. Higher Drd2 levels in A02 cerebella than in WT cerebella might be caused by the 50-fold higher levels of expression of the WT human SCA1 allele (100 transgene copies) in PC of A02 mice when compared with the endogenous levels in WT mouse cerebellum (26). Higher Drd2 levels in A02 mouse cerebellum, but not of Calb1 or β-actin, suggest that cerebellar Drd2 expression in mice might be regulated by Atxn1. In contrast to A02 mice, Drd2 levels are significantly reduced in cerebellum from B05 mice when compared with cerebella of A02 (P < 0.001) and WT (P = 0.029) mice. Importantly, the levels of soluble mutant Atxn1[82Q] in B05 mouse cerebellum are similar to those of Atxn1[30Q] in A02 mouse cerebellum (P = 0.122) and endogenous murine Atxn1[2Q] (P = 0.328). It is important to note that reduced Drd2 and Calbindin D-28K (Calb1) levels detected in the cerebellum of 5-week-old B05 mice occur in the absence of apparent neuroanatomical changes (27). The significant 1.25-fold reduction in the Drd2 levels in B05 mouse cerebellum when compared with A02 mouse cerebellum indicates that the polyQ expansion diminishes the effects of Atxn1 on Drd2 expression.

Atxn1 functions synergistically with Sp1 to co-regulate Drd2 expression

On the basis of the observations that Drd2 expression is altered in mice lacking Atxn1 and in transgenic mice expressing human Atxn1 with either 30 or 82 glutamines (Fig. 3), we examined whether Atxn1 transregulates Drd2 expression and investigated the underlying mechanisms. Luciferase assays in human neuroblastoma dopaminergic SH-SY5Y cells with the proximal sequence of the rat Drd2 promoter upstream of
the luciferase reporter gene (28) were established to examine whether Atxn1 regulates Drd2 transcription. Because the AXH module is also present in HBP1 transcription factor (6,7) and it has been proposed to play a role in transcription (10,16), we also examined the effects of isolated AXH (29) on the expression of the reporter gene linked to the Drd2 promoter (Fig. 4A). Our results clearly show that the region spanning the AXH domain in Atxn1 (amin acid 570–689) transactivates expression of the reporter gene targeting the Drd2 promoter (F3,8 = 295.103, P < 0.001). Significant 1.6- and 5.4-fold increase activations of the rat Drd2 promoter by the AXH domain were observed with 2 and 4 μg of DNA, respectively (2/0 μg, P = 0.024; 4/0 μg, P < 0.001). Consistently, we identified 19 HBP1-binding sites in the Drd2 promoter. While the transactivation effects on the Drd2 promoter by full-length WT Atxn1[30Q] were not as evident as those observed with the AXH domain, a significant 1.26-fold activation by Atxn1[30Q] was detected at the highest (4 μg) DNA concentration (F3,12 = 12.99, comparison with control, P = 0.002; Fig. 4B). The transactivation effects of the reporter gene mediated by Atxn1 through the Drd2 promoter were significantly abrogated by the expanded polyQ (F3,12 = 96.72, post-hoc tests: 2/0 μg, P = 0.001; 4/0 μg, P = 0.001; Fig. 4C). Altogether, these observations demonstrate that both the AXH domain in Atxn1 and full-length WT Atxn1 co-regulate Drd2 transcription, and these effects are abrogated by the expanded polyQ in Atxn1.

Because our microarray analysis identified significant down-regulation of Sp1 mRNA levels in Atxn1-null mice, and Drd2 expression is transregulated by Sp1 through several Sp1-binding sites located within the Drd2 promoter (30), we next examined the effects of both Atxn1 and Sp1 on regulating the expression of the reporter gene from the Drd2 promoter. As expected, Sp1 strongly transactivated expression of the reporter gene. 1.8- and 13.4-fold increase activations through the rat Drd2 promoter were observed with 2 and 4 μg DNA vector expressing Sp1, respectively (F3,12 = 3599.43, post-hoc tests: 2/0 μg, P = 0.02; 4/0 μg, P < 0.001; Fig. 4D). When neuroblastoma cells were co-transfected with 2 μg of each DNA construct containing the isolated AXH domain of Atxn1 and Sp1, an 8.3-fold synergistic activation of the reporter gene was detected (F5,18 = 78.71, P = 0.001; Fig. 5A). These results indicate cooperation of Sp1 and the AXH module on regulating Drd2 transcription since the 8.3-fold increase Drd2 transactivation by both Sp1 and AXH is not explained by summing the
individual activation effects on Drd2 transcription by Sp1 (1.8-fold) and AXH (1.6-fold). Synergistic Drd2 transactivation is also observed with both full-length Atxn1[30Q] and Sp1 (3.5-fold, \( P = 0.045 \)), and the effects are abrogated by the expanded polyQ (\( P = 0.04 \)). The absence of the AXH domain within Atxn1 (amino acid 570–689) abrogates the effects on Drd2 Sp1-dependent transactivation by Atxn1 (\( P = 0.004 \)). Overall, these results demonstrate that Atxn1 co-regulates Drd2 Sp1-dependent transcription and that the AXH module of Atxn1 is required for Atxn1 transcriptional co-regulation. The effects of Atxn1 co-regulating Drd2 Sp1-dependent transactivation are abrogated by the expanded polyQ in Atxn1.

Atxn1 interacts with Sp1 through the AXH domain and the interaction is modulated by the polyQ tract

On the basis of the effects of Atxn1 on Drd2 Sp1-dependent transactivation, we next examined whether Atxn1 exerts these effects through interaction with Sp1. Tandem affinity purifications (TAP) of WT Atxn1[30Q] or mutant Atxn1[82Q] from SH-SY5Y cells show interaction of Atxn1 with endogenous Sp1 (Fig. 6A). The Atxn1–Sp1 interaction is weaker when Atxn1 contains an expanded polyQ (0.54-fold, \( P = 0.012 \)). TAP of Atxn1 also showed interaction with exogenously expressed Sp1 in SH-SY5Y cells (Supplementary Material, Fig. S3). Furthermore, Sp1 co-immunoprecipitates with either...
WT or mutant Atxn1 when they are co-expressed in neuroblastoma cells (Fig. 6B), although reduced Sp1 molecules co-immunoprecipitate with mutant Atxn1[82Q] than with WT Atxn1[30Q] (0.43-fold, \( P = 0.0295 \)). Interestingly, stronger Sp1-interaction is detected with the AXH domain than with either Atxn1[30Q] (0.44-fold, \( P = 0.0025 \)) or Atxn1[82Q] (0.19-fold, \( P = 0.011 \)). This confirms that the polyQ tract modulates the Atxn1–Sp1 interaction. Furthermore, the AXH domain within Atxn1 is required for Sp1 co-immunoprecipitation since Sp1 did not co-immunoprecipitate with Atxn1 lacking the AXH domain. This demonstrates that the Atxn1–Sp1 interaction is mediated by the AXH module within Atxn1.

Figure 4. Transcriptional regulation of the rat Drd2 promoter by Atxn1 in human neuroblastoma SH-SY5Y cells. SH-SY5Y cells were co-transfected with variable DNA concentrations (1, 2 or 4 \( \mu \)g) of CMV vector expressing the AXH domain of Atxn1 (amino acid 570–689) (A), WT human Atxn1[30Q] (B), mutant human Atxn1[82Q] (C) or transcription factor Sp1 (D), and vector DNA containing the firefly luciferase reporter gene under the control of the rat Drd2 promoter. Dual-reporter (firefly and renilla luciferases) fold activations were measured and compared with normalized control values. The isolated AXH domain of Atxn1 (A), Atxn1[30Q] (B) or Sp1 (D) transactivate the Drd2 promoter, whereas the expanded polyglutamine diminishes the effects of Atxn1 on transcription (C). Significant 1.6- and 5.4-fold increase activations of the rat Drd2 promoter by the AXH domain are observed with 2 and 4 \( \mu \)g of DNA, respectively [2/0 \( \mu \)g: \( P = 0.024 \); 4/0 \( \mu \)g: \( P < 0.001 \); (A)]. A 1.26-fold increase activation by Atxn1[30Q] is detected with 4 \( \mu \)g of DNA [4/0 \( \mu \)g: \( P = 0.002 \); (B)]. The transactivation effects are significantly abrogated by the expanded polyglutamine [2/0 \( \mu \)g, \( P = 0.001 \); 4/0 \( \mu \)g, \( P = 0.001 \); (C)]. Sp1 strongly transactivates expression of the reporter gene: 1.8- and 13.4-fold increase activations through the rat Drd2 promoter are observed with 2 and 4 \( \mu \)g DNA vector expressing Sp1, respectively [2/0 \( \mu \)g, \( P = 0.02 \); 4/0 \( \mu \)g, \( P < 0.001 \); (D)]. Corresponding immunoblots are shown on the bottom of each panel where \( \beta \)-actin levels reveal equal protein loading. Atxn1[30Q], Atxn1[82Q] and Sp1 are detected with antibodies that also recognize endogenous corresponding proteins present in control lanes (0 \( \mu \)g). Data are shown as mean \( \pm \) SEM for three independent experiments performed with each expression vector. \(* P < 0.05; ** P < 0.01\). Bars correspond to SEM.
Atxn1 occupancy at the Drd2 promoter in mouse cerebellum

Chromatin immunoprecipitation (ChIP) assays using formaldehyde cross-linked chromatin extracts prepared from human neuroblastoma SH-SY5Y cells reveal occupancy of either the AXH domain, WT Atxn1[30Q] or mutant Atxn1[82Q] at the rat Drd2 promoter (Fig. 7A). Reduced Drd2-promoter occupancy detected by decreased amounts of PCR product yielded after three independent amplifications, was noted with mutant Atxn1[82Q] when compared with WT Atxn1[30Q]. This is indicative of a close association of Atxn1 with the Drd2 promoter through its AXH domain and suggests that the Atxn1 occupancy is modulated by the expanded polyQ in mutant Atxn1.

Figure 5. Functional synergistic effects of Atxn1 on Sp1-dependent transactivation of the rat Drd2 promoter in SH-SY5Y cells. (A) Effects of AXH (amino acid 570–689), WT Atxn1[30Q], mutant Atxn1[82Q] and Atxn1 with a deletion of the AXH domain Atxn1[30Q]ΔAXH on Drd2 Sp1-dependent transactivation (using 2 µg of each plasmid). A 8.3-fold synergistic activation of the reporter gene is detected (P = 0.001) with AXH and Sp1. Synergistic Drd2 transactivation is also observed with both full-length Atxn1[30Q] and Sp1 (3.5-fold, P = 0.045) and the effects are abrogated by the expanded polyglutamine (P = 0.04). The absence of the AXH domain within Atxn1 abrogates the effects on Drd2 Sp1-dependent transactivation by Atxn1 (P = 0.004). (B) Corresponding immunoblots are shown where β-actin levels reveal equal protein loading. Data are shown as mean ± SEM for three independent experiments performed for each condition. **P < 0.05; ***P < 0.01. Bars correspond to SEM.

ChIP experiments with chromatin extracts prepared from 14-day-old WT mouse cerebellum demonstrated Atxn1 occupancy at the murine Drd2 promoter in vivo (Fig. 7B). Similar experiments with extracts from Sca1 KO cerebella did not reveal precipitation of the Drd2 promoter illustrating specificity of the assays. Interestingly, ChIP assays with αSp1 antibodies precipitated Drd2 promoter sequences from both WT and KO mice cerebella, although greater quantities of resulting PCR products were consistently detected in independent PCR amplifications with two different amounts of immunoprecipitated input DNA from three WT mouse cerebellar samples, when compared with KO mice samples (data not shown). No amplifications occurred with control non-specific αIgG antibodies and no occupancies of Atxn1 and Sp1 at the β-actin promoter were revealed in ChIP assays demonstrating specific occupancies of both Atxn1 and Sp1 at the Drd2 promoter. Reduced Sp1 occupancy at the Drd2 promoter in Sca1 KO mouse cerebellum along with the reduced Drd2 mRNA and protein levels detected in the KO mouse cerebella, the synergistic activation of the Drd2 promoter by Atxn1 and

Figure 6. Atxn1 interacts with Sp1 through the AXH domain and the interaction is abrogated by the polyglutamine expansion in Atxn1. (A) TAP of WT Atxn1[30Q], mutant Atxn1[82Q] or control vector (C) from SH-SY5Y cells show association of Atxn1 with Sp1. Input fractions (5%) and equal quantities of eluted protein were analysed by immunoblotting with αAtxn1 11750V and αSp1 antibodies. Sp1 associates more efficiently with WT Atxn1[30Q] than with mutant Atxn1[82Q] (0.54-fold, P = 0.0117), and no Sp1 is detected in purifications of the control vector (C). (B) Co-immunoprecipitations of Sp1 and Atxn1 in SH-SY5Y cells expressing Sp1 and either the AXH domain, WT Atxn1[30Q], mutant Atxn1[82Q] or Atxn1 lacking the AXH domain Atxn1[30Q]ΔAXH. Control experiments were performed with non-specific antibodies (C). Sp1 co-immunoprecipitates with either WT or mutant Atxn1 when they are co-expressed in neuroblastoma cells, although reduced Sp1 molecules co-immunoprecipitate with mutant Atxn1[82Q] than with WT Atxn1[30Q] (0.43-fold, P = 0.0295). Interestingly, stronger Sp1-interaction is detected with the AXH domain than with either Atxn1[30Q] (0.84-fold, P = 0.0025) or Atxn1[82Q] (0.19-fold, P = 0.011). This demonstrates that the polyQ tract modulates the Atxn1-Sp1 interaction, and that the AXH domain within Atxn1 is required for Sp1 co-immunoprecipitation since Sp1 did not co-immunoprecipitate with Atxn1 lacking the AXH domain (Atxn1[30Q]ΔAXH).

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Figure 7. ChiP assays with formaldehyde cross-linked chromatin extracts from human neuroblastoma SH-SY5Y cells and mouse cerebellum reveal Atxn1 occupancy at the Drd2 promoter. (A) Plasmid DNA fragments spanning three regions of the rat Drd2 promoter (D2-1: from −835 to −684 bp; D2-2: from −455 to −307 bp; D2-3: from −144 to +25 bp) were co-immunoprecipitated, as shown by PCR amplification with specific primers, with the AXH domain, WT human Atxn1[30Q] or mutant human Atxn1[82Q], demonstrating occupancy of the rat Drd2 promoter by Atxn1 in neuroblastoma cells. (B) Genomic DNA fragments spanning the mouse Drd2 promoter (from −172 to −8 bp) from mouse cerebellum were immunoprecipitated using αAtxn1 11750V, αSp1 or αRNA-pol-II antibodies and PCR amplified with specific primers (left panel). Chromatin DNA fragments spanning the mouse Drd2 promoter are not immunoprecipitated using chromatin extracts from KO mouse cerebellum with αAtxn1 antibodies as revealed by negative PCR amplifications. This demonstrates occupancies of Atxn1, Sp1 and RNA-pol-II at the Drd2 promoter in mouse cerebellum. In contrast, PCR amplifications from immunoprecipitated chromatin spanning the Drd2 promoter with αSp1 antibodies are less efficient, as revealed by PCR product yield, from KO mouse cerebellum than from WT. This suggests reduced Sp1 occupancy at the Drd2 promoter in KO mouse cerebellum. αIgG antibodies were used as non-specific controls. Chromatin spanning the β-actin promoter is not immunoprecipitated with αAtxn1, αSp1 or αIgG antibodies, as shown by PCR amplification, but it is immunoprecipitated with αRNA-pol-II antibodies, illustrating specificity of the ChiP assays (right panel). Fragment sizes (in base pair) of PCR products are indicated on the right of each panel.

Sp1 in SH-SY5Y cells (Fig. 5) and the interaction between Atxn1 and Sp1 are indicative of possible alterations in transcriptional activity of the Drd2 gene in Atxn1-null mice.

DISCUSSION

The main goal of this study is to gain insights into the molecular mechanisms underlying cerebellar dysfunction mediated by loss of Atxn1 function in Atxn1-null mice. Thus, we examined the cerebellar gene expression profiles of Sca1−/− mice at 5-weeks of age and identified changes in Atxn1-null mice cerebella in expression of genes implicated in translational initiation, Wnt-signalling, retinol binding, nucleic acid binding and intracellular signalling cascades. Remarkably, our microarray analysis identified alterations in gene expression of the related zinc-finger transcription factors Sp1 and Kruppel-like factors 4 and 9, which bind GC-rich DNA elements to regulate transcription. Consistently, we also detected dysregulation of expression of several genes with Sp1-binding sites within their promoters. Some genes dysregulated by loss of Atxn1 function are implicated in Wnt-signalling. This evidence together with the fact that HBPI transcription factor regulates Wnt-signalling through its AXH domain (31) provide new evidence linking Atxn1 and its AXH module in Wnt-signalling. Wnt-signalling interacts with RA-signalling to regulate neurogenesis, neuronal differentiation and specification (32,33). Alterations in Wnt-receptor- and RA-signalling in Atxn1-null mice suggest that Atxn1 might regulate genetic programs required for neuronal specification during development. This is supported by the recent data evidencing the influence of Atxn1 during cerebellar development through RA-signalling (18).

Our study has identified dysregulation of neurogranin (Nrgn) in Sca1−/− mice. Interestingly, while Nrgn expression is up-regulated in B05 transgenic mice expressing human expanded Atxn1 in cerebellum (12), Nrgn levels are down-regulated in Atxn1-null mice. Nrgn is a brain-specific post-synaptically located protein kinase C substrate that binds to calmodulin in the absence of calcium and is a target of thyroid hormone triiodothyronine (T3) and RA in the brain. Ablation of Nrgn expression in mice impairs spatial learning and causes alterations in hippocampal short-term plasticity (34). Therefore, Nrgn down-regulation in Atxn1-null mice might underlie the learning and memory deficits and the hippocampal short-term synaptic plasticity alterations led by loss of Atxn1 function. Alterations in levels of Nrgn, Drd2, calcium sensors and genes controlled by retinoic acid-signalling in both Atxn1-null and SCA1 transgenic mice suggest that the expanded polyQ in SCA1 might dysregulate molecular pathways that are regulated by Atxn1 in the mouse brain. Our luciferase assays showing transcription abrogation under the control of the Drd2 promoter by mutant Atxn1 support this hypothesis.

Our study also shows significant Drd2 down-regulation in the cerebella of mice lacking Atxn1 and B05 transgenic mice expressing mutant human ataxin with 82 glutamines in PC. The Drd2 receptor is a Gαo-protein coupled receptor of the D2-like receptor subfamily that regulates a myriad of intracellular signalling pathways (35). Among all five DA receptors, Drd2 shows the highest affinity for DA, which is now recognized as a neuromodulator of synaptic transmission. Pre-synaptic and post-synaptic Drd2 receptors control DA release in dopaminergic neurons and mediate events in the target cells in areas receiving dopaminergic innervation, respectively. Drd2 stimulation inhibits adenylate cyclase, via their Gαi/o-protein subunit, preventing cyclic adenosine 3’,5’-cyclic phosphate (cAMP) synthesis. This reduces protein kinase A (PKA) activity resulting in decreased phosphorylation of cyclic AMP-dependent PKA substrates, such as the DA- and cAMP-regulated phosphoprotein, M₆ 32 kDa (DARPP-32). Through cAMP-independent pathways, as a result of the receptor-induced liberation of Gβγ subunits, Drd2 stimulation in response to DA also leads to specific dephosphorylation and inactivation of Akt activity, and concomitant activation of its substrate glycogen synthase kinase 3 (GSK-3). Drd2 receptors also modulate
ion channels, phospholipases, receptor tyrosine kinases, glutamatergic and GABAergic neurotransmission, and stimulate MAP kinase pathways. Remarkably, decreased expression of Drd2 in flies and mice result in reduced locomotor activities, associative deficits, altered drug-responses and modification of the electrophysiological properties (36,37). In the cerebellar cortex, dopaminergic projections from the A10 dopaminergic cell group of the ventral tegmental area (VTA) in the midbrain have been identified, and Purkinje neurons show the most DA receptor protein immunoreactivity (reviewed in 38). A direct connection exists between the deep cerebellar nuclei and the mesencephalic dopaminergic system (39,40). Indeed, large soma cells of the interposed and dentate nuclei project to the contralateral SNpc and VTA, and cells from fastigial nuclei project to the ipsilateral dopaminergic mesencephalon. Biochemical studies in the cerebellum have demonstrated active re-uptake of DA into cerebellar synaptosomes, release of endogenous DA by microdialysis and the presence of Drd2 receptors. This and other evidence led to propose that DA might regulate the responsiveness of PCs to GABA through cAMP cascades (41,42). Therefore, DA could be implicated in the modulation of two forms of synaptic plasticity in the cerebellar PC synapse: rebound potentiation and long-term depression, which are two key processes for the regulation of cerebellar functions. Loss or impaired function of Drd2 receptors in PC could impair directly the feedback inhibition of their activity mediated by DA released from their dendrites. It will might affect glutamate- and GABA(A)-receptor mediated signalling in the cerebellar cortex and, indirectly, disrupt the balance of inhibitory inputs by basket cells and satellite cells, and excitatory inputs by glutamatergic granule cells, parallel fibres and climbing fibres influencing PC output. Interestingly, PC degeneration (Nna1pcd, pcd) mutant mice show alterations in cerebellar Dat/Sle6a3 and Drd2 levels (43). A significant increase of Dat/Sle6a3 levels in the deep cerebellar nuclei and decreased levels of both Dat/Sle6a3 and Drd2 are detected in the cerebellar molecular layer in these mice. Nna1pcd mutant mice are mainly charac-
terized by the complete, primary loss of the PCs and the secondary, partial, retrograde loss of the granule and inferior olive neurons, and are considered a model of human degenerative ataxia. It is thought that there is an increased excitatory input on the dopaminergic mesencephalic neurons and alterations of dopaminergic neurotransmission in basal ganglia, cortical and limbic regions in Nna1pcd mutant mice. Altogether, these observations highlight the conserved role of Drd2-signalling in regulating motor behaviours and some forms of learning and memory, and point to possible alterations of Drd2-dependent functions underlying the neurobehavioural deficits in Atxn1-null mice and some motor deficits in SCA1 transgenic mice.

An important contribution of this study is to demonstrate the role of Atxn1 and its AXH module in co-regulating transcription. Atxn1 co-regulates Sp1-dependent transcription occupying at the Drd2 promoter in mouse cerebellum and the transactivation effects are exerted by the AXH domain within Atxn1, which are abrogated by the expanded polyQ in mutant Atxn1. Furthermore, Atxn1 interacts with Sp1 through the AXH domain and the interaction most likely contributes to the effects of Atxn1 on transcriptional regulation since the polyQ expansion abrogates the Atxn1–Sp1 interaction and Drd2 Sp1-dependent transcription. The fact that the AXH domain is required for the Atxn1–Sp1 association suggests a physical interaction between Atxn1 and Sp1. This is supported by the proposed role for the AXH domain within Atxn1 in protein–protein binding (29,44). Interestingly, Drd2 is down-regulated in the striatum of pre-symptomatic patients with Huntington’s disease (HD) (45,46), a motor neurodegenerative disease caused by an expanded polyQ in huntingtin. Drd2 dysregulation in HD appears to be mediated by interference of the expanded polyQ with interactions of huntingtin with Sp1, transcriptional co-activators and components of the core transcription apparatus (47,48). Importantly, treatment of HD transgenic mice with the D2-like receptor agonist quinpirole reduces excitotoxicity and re-establishes neuronal function and recovery of synaptic plasticity (49). Overall, these findings support the contribution of Drd2-signalling in motor functions, suggesting that dysregulation of common genetic programs might be an early molecular event in movement disorders caused by expanded polyQ, and offer the possibility of modulating Drd2-dopaminergic signalling in therapeutic strategies to re-establish neuronal function in SCA1.

In summary, using microarray analysis, we have identified the genetic programs that are regulated by Atxn1 in mouse cerebellum, which might underlie the motor and neurobehavioural deficits in Sca1-null mice. A selective set of genes regulated by Sp1-dependent transcription and the RA/thyroid hormones and Wnt-receptor signalling pathways are dysregulated in mice lacking Atxn1. Drd2 levels are significantly reduced in the cerebella of null mice lacking Atxn1 and SCA1 transgenic mice, suggesting that common signalling pathways are dysregulated in both mouse models. This evidence has important implications for understanding SCA1 pathogenesis. We provide evidence for transcriptional regulation by Atxn1 and demonstrate that Atxn1 interacts and functions synergistically with Sp1 to co-regulate Drd2 expression. Therefore, this study provides new evidence implicating Atxn1 in co-regulating Sp1-dependent transcription and identifies novel molecular targets regulated by Atxn1 that may underlie some motor deficits in SCA1. Identifying the molecular pathways regulated, either directly or indirectly, by Atxn1-mediating cerebellar function can provide insights into the early molecular mechanisms underlying cerebellar dysfunction in SCA1 that could be used to design therapeutic strategies to prevent neuronal dysfunction and motor deficits at pre-symptomatic stages of the disease in SCA1 patients.

MATERIALS AND METHODS

Mice

Inbred 129/SvEv Atxn1-null mice are described in detail elsewhere (5). All animal procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986 of the UK and were approved by appropriate Ethical Committees established by the UCL Institute of Child Health. Mouse genotyping protocols are included in the Supplementary Material.
Tissue harvesting, RNA extraction and microarray analysis

Cerebellar expression profiles were generated from 5-week-old male 129/SvEv mice and compared with age- and gender-matched WT control littermates. Sample preparation and processing procedures were performed as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix Inc., Santa Clara, CA). A detailed protocol is included in the Supplementary Material. Microarray MIAME-compliant data is deposited in the ArrayExpress database (www.ebi.ac.uk/miamexpress).

Semi-quantitative real-time RT–PCR

Q-RT–PCR was used to validate changes in the expression of a subset of 10 genes identified in our microarray analysis with 5 WT and 5 KO mice cerebellar samples. We performed a single step reverse transcriptase PCR reaction (Qiagen Inc., Hilden, Germany) with a DNA Engine Opticon (Bio-Rad, Hercules, CA), continuous fluorescence detection and SYBR green fluorescence reagents as specified by the manufacturer on 200 ng RNA target in a final reaction volume of 50 µl. Primer sequences were designed for each target gene containing minimal internal structures (i.e. hairpins and primer dimers) and optimal melting temperatures with each Tm within 1°C of the other using QuantiProbe software (Qiagen Inc., Hilden, Germany) to target splice sites within the RNA sequence. The primer sequences and conditions used in Q-RT–PCR experiments are described in the Supplementary Material. Samples were compared using the relative comparative Ct method (50).

Promoter analysis

The Transcriptional Regulatory Element Database (TRED) (51) was searched to obtain the promoter sequences of the genes identified in this study. Retrieved sequences were subsequently analysed with JASPAR (52) and ConSite (53) to obtain DNA-binding profiles for Sp1, HBp1 and RA/thyroid hormone receptor.

Protein extraction and immunoblotting

Proteins from mouse brains/cerebella were extracted by homogenization in lysis buffer containing 8 M urea, 2 M thiourea, 4% 3-[3-cholamidopropyl]dimethylammonio]-1-propane-sulfonate and 0.5% Nonidet P40 (NP-40) supplemented with protease inhibitors Complete (Roche, Basel, Switzerland). Homogenates were centrifuged at 17 500g for 15 min at 4°C. The resulting supernatants were diluted 5-fold with RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) and mixed with 5X SDS-sample buffer (625 mM Tris pH 6.8, 10% SDS, 50% glycerol, 10% β-mercaptoethanol and bromophenol blue). Samples were heated at 95°C for 5 min. Cell extracts from SH-SY5Y cells were prepared in passive lysis buffer (Promega, Southampton, UK) as described in the manufacturer’s instructions and treated as earlier. Following electrophoresis and transfer to nitrocellulose membrane (Protran, Schleicher and Schuell, Middlesex, UK), membranes were probed with primary antibodies recognizing Drd2 (sc-5303, Santa Cruz Biotechnologies, Santa Cruz, CA), Sp1 (Santa Cruz Biotechnologies, Santa Cruz, CA), β-actin AC15, Flag M2, C-Myc (9E10) and Calb1 (all from Sigma-Aldrich, Dorset, UK), or Atxn1 (11750V; 54). Peroxidase-conjugated secondary antibodies were detected by enhanced chemiluminescence (GE Healthcare, Uppsala, Sweden). Immunoblots were quantified using a GS-800 calibrated densitometer and Quantity one software (Bio-Rad, Hercules, CA).

Cell culture and dual-reporter luciferase assays

Human dopaminergic neuroblastoma SH-SY5Y cells were obtained from the European Collection of Cell Cultures (ECACC-Sigma Aldrich, Dorset, UK). SH-SY5Y cells were maintained in Ham’s F12; Earle’s minimal essential medium EMEM (1:1) containing 2 mM Glutamine, 1% non-essential amino acids, 15% Foetal Bovine Serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells from passages 14–20 were used and they were seeded at 60–70% confluency into 30 mm six-well dishes. Cells were transfected in six-well plates using Lipofectamine 2000 (Invitrogen, Paisley, UK) according to the manufacturer’s instructions. cDNAs corresponding to Atxn1[30Q], Atxn1[82Q] and Atxn1 lacking the AXH domain (amino acid 570–689) (Atxn1[30Q]ΔAXH) were subcloned into the CMV-tag1 vector (Stratagene, La Jolla, CA), the Atxn1 AXH domain (amino acid 570–689) subcloned into pEF/C/Myc/Nuc vector (29), and Sp1 within the pEVR2 vector (55). DNAs (1–4 µg) were co-transfected with either the rat Drd2 promoter region (from −850 to +133 bp relative to the transcription start site) inserted into the pGL3 luciferase reporter vector (pGL3-Drd2) (28) or with empty pGL3 vector (2 µg), and the control renilla luciferase vector (0.2 µg). DNA concentrations were equalized with empty vector (CMV-Tag1) and normalized control values represent luciferase activity obtained when CMV-tag1 is co-transfected with reporter vector. Cells were harvested 48 h after transfection in passive lysis buffer (Promega, Southampton, UK) and luciferase activities were determined using the dual luciferase assay system (Promega, Southampton, UK) according to the manufacturer’s instructions. Results are expressed as a ratio of the firefly/renilla luciferase activities except for Sp1 co-transfections where firefly luciferase activity was normalized to the Sp1 protein expression levels determined by immunoblotting with αSp1 antibodies as described earlier.

TAP and co-immunoprecipitation

pNTAPB-Atxn1[30Q] and pNTAPB-Atxn1[82Q] were generated by subcloning the Atxn1 30Q and 82Q cDNAs (13) into the EcoRI and Xhol sites of the pNTAPB vector (Stratagene, La Jolla, CA). Ten micrograms of pNTAPB, pNTAPB-Atxn1[30Q] or pNTAPB-Atxn1[82Q] DNAs were transfected into SH-SY5Y cells at 60% confluence in 10 cm culture dishes using Lipofectamine 2000 and OptiMEM medium (Invitrogen, Paisley, UK) according to the manufacturer’s instructions. Forty-eight hours after transfection, the culture medium was replaced with SH-SY5Y culture medium (described earlier).

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Chromatin immunoprecipitation

SH-SY5Y cells were co-transfected with 10 μg vector DNA of Flag-Atxn1[30Q], Flag-Atxn1[82Q] or Myc-AXH, and pGL3-Drd2 (from −850 to +133 relative to the transcription start site) as described earlier. Forty-eight hours after transfection, cells were harvested and processed as described (56). Immunoprecipitations were carried out using 5 μg of αMyc, αFlag, or control serum. Input and immunoprecipitated DNAs were subjected to PCR amplification with the primers and conditions included in the Supplementary Material. For in vivo ChIP assays, whole cerebella from 14-day-old WT and KO mice were dissected out and cut into 50–100 mg pieces. Cerebellar tissues were washed once with ice-cold PBS containing protease inhibitors (PBS/Pi), resuspended in 600 ml PBS/Pi containing 1% formaldehyde, incubated at room temperature for 15 min, centrifuged at 3000 g and washed with PBS/Pi. Further processing was done using EZ ChIP kit components (Upstate Biotechnologies, Temecula, CA) as described in the manufacturer’s protocol and in Supplementary Material. PCR amplifications were performed with two DNA input volumes and conditions chosen that did not saturate the PCR reaction. Briefly, chromatin extracts from three cerebellar WT and KO samples were amplified independently in parallel using two DNA amounts as input in the PCR reaction (1 and 2 μl). Primers and conditions are described in the Supplementary Material.

Statistics

Statistical analyses were performed with SPSS 11.0 (Chicago, IL). Data were tested for normal distribution and equality of variances using the Shapiro–Wilks’ W-test and the Levene’s test of homogeneity of variances, respectively. Normally distributed data were analysed by one-way ANOVA followed by post-hoc comparisons with either the Tukey–Kramer honestly significant different test for groups of data with equal variances or, alternatively, with the Games–Howell test in one-way ANOVA analyses with unequal variances. Statistical significance was defined as a P-value < 0.05.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

REFERENCES


