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Alternative Splicing Events Are a Late Feature of Pathology in a Mouse Model of Spinal Muscular Atrophy

Dirk Bäumer¹, Sheena Lee¹, George Nicholson², Joanna L. Davies², Nicholas J. Parkinson¹, Lyndsay M. Murray³, Thomas H. Gillingwater³, Olaf Ansorge⁴, Kay E. Davies¹, Kevin Talbot¹,5*

Abstract

Spinal muscular atrophy is a severe motor neuron disease caused by inactivating mutations in the SMN1 gene leading to reduced levels of full-length functional SMN protein. SMN is a critical mediator of spliceosomal protein assembly, and complete loss or drastic reduction in protein leads to loss of cell viability. However, the reason for selective motor neuron degeneration when SMN is reduced to levels which are tolerated by all other cell types is not currently understood. Widespread splicing abnormalities have recently been reported at end-stage in a mouse model of SMA, leading to the proposition that disruption of efficient splicing is the primary mechanism of motor neuron death. However, it remains unclear whether splicing abnormalities are present during early stages of the disease, which would be a requirement for a direct role in disease pathogenesis. We performed exon-array analysis of RNA from SMN deficient mouse spinal cord at 3 time points, pre-symptomatic (P1), early symptomatic (P7), and late-symptomatic (P13). Compared to littermate control mice, SMA mice showed a time-dependent increase in the number of exons showing differential expression, with minimal differences between genotypes at P1 and P7, but substantial variation in late-symptomatic (P13) mice. Gene ontology analysis revealed differences in pathways associated with neuronal development as well as cellular injury. Validation of selected targets by RT–PCR confirmed the array findings and was in keeping with a shift between physiologically occurring mRNA isoforms. We conclude that the majority of splicing changes occur late in SMA and may represent a secondary effect of cell injury, though we cannot rule out significant early changes in a small number of transcripts crucial to motor neuron survival.

Introduction

Autosomal recessive Spinal Muscular Atrophy (SMA) is a leading genetic cause of infant mortality, with a carrier frequency of 1:50 and an annual incidence of 1 in 10,000 live births [1]. Affected individuals develop symmetrical, proximal weakness resulting from neurogenic muscle atrophy, and ultimately leading, in the most severely affected individuals, to death from respiratory failure. The pathological correlate of these symptoms is selective loss of large alpha motor neurons in the ventral horn of the spinal cord. The vast majority of cases are caused by homozygous deletion of the survival motor neuron 1 (SMN1) gene [2] with subsequent reduction in levels of the SMN protein [3]. SMN is highly conserved in evolution and ubiquitously expressed. Complete loss of SMN, which is incompatible with life [4], is prevented by production of SMN from the SMN2 gene, a near identical parologue of SMN1 which has arisen from an inverted duplication event in recent evolution. The presence of a translationally silent C-T transition in SMN2 exon 7, however, leads to disruption of an exonic splice-enhancer (ESE) element and exon 7 skipping in the majority of SMN2 derived transcripts [5,6]. The functional consequence is that SMN2 produces only very little full length SMN (FL-SMN), while the SMNΔ7 isoform is translated into an unstable protein that is rapidly degraded [7]. Disease severity is broadly proportional to residual SMN levels, which is a function of SMN2 copy number, although other modifying factors are involved in some cases [8,9].

The mechanism by which SMN deficiency leads to selective lower motor neuron loss in SMA is poorly understood and difficult to reconcile with its ubiquitous expression unless either SMN has a motor neuron-specific function or motor neurons are selectively vulnerable to a deficiency in the general function of SMN common to all cells. Currently, the best characterised function of SMN is as part of a multi-protein complex which is critical for the core steps in the assembly of small nuclear ribonuclear proteins (snRNPs), components of the spliceosome, the cellular machinery that controls splicing of pre-mRNAs [10,11]. In particular, SMN acts in the cytoplasmic assembly of Sm core proteins on snRNAs, which is a prerequisite for import of snRNPs into the nucleus [12]. SMN levels and the activity of snRNP assembly vary during

References

[1]...[12]
development and according to tissue type. In the mouse spinal cord, snRNP assembly is highest during embryogenesis and early postnatal development and then falls to a baseline level when myelination occurs [13]. Mouse models of SMA, which have reduced SMN levels, show a drop in snRNP assembly activity as measured by in vitro assays, while steady state snRNP levels measured in tissues are only mildly reduced [14]. Interestingly, a subset of snRNPs belonging to the minor spliceosome seems to be differentially affected [14,15]. Several strands of evidence support the notion that reduced snRNP assembly is associated with motor neuron degeneration. The subtle motor neuron loss that has been reported by some investigators at late stages in mice heterozygous null for Smn can be accelerated by crossing with mice heterozygous null for Gemin2, another core component of the SMN complex. This is associated with a reduction in snRNP assembly [16]. In zebrafish, a failure of embryonic motor axon growth can be induced by silencing not only SMN, but also gemin2. The observed defects can be rescued by direct injection of U snRNPs [17]. However, other studies in zebrafish indicate that the axonal degeneration phenotype is not coupled to U snRNP assembly [18]. Reducing SMN levels in HeLa cells by RNAi leads to an increased error rate in splice-site pairing, which has also been observed in fibroblasts from SMA patients [19]. Finally, a recent study in SMA mice found that at end-stage, widespread splicing abnormalities can be found in several tissues including the spinal cord. Importantly, different transcripts were found to be altered in a tissue-specific manner [15].

While both snRNP assembly dysfunction and splicing abnormalities have been documented in models of SMA, several questions remain. It is still unclear whether splicing abnormalities cause motor neuron loss in SMA, or whether they are a late occurrence in disease, either as a consequence of spliceosome dysfunction or the severe physiological alterations secondary to respiratory distress, hypoxia and malnutrition. If spliceosome dysfunction is critical for disease pathogenesis, the mechanism of splicing alterations needs to be further delineated. In addition, the role of SMN in spliceosome assembly is only one of many functions that are potentially altered in SMA, including roles in transcription regulation [20–22], axonal transport of mRNA and RNPs [23,24] as well as regulation of local translation at the neuromuscular junction [25].

This study had two aims. First, we wanted to address the question of whether abnormal splicing events are a cause or consequence of SMA. We utilised an exon-specific microarray to examine the transcriptome of spinal cord samples harvested from SMA and control mice at pre-symptomatic, early symptomatic and late-symptomatic stages, to test the hypothesis that widespread alteration of splicing precedes disease onset. Secondly, we used the difference in temporal mRNA expression pattern between SMA and control mice to identify neuronal pathways disturbed by SMN deficiency.

Results

Correlation of mouse phenotype and motor neuron loss

The mouse model used in this study (Smn−/−; Smn2; SmnA7) is the most commonly used model of severe SMA and has been described previously [26]. The maximum lifespan of SMA mice was 14 days. Subtle differences in weight compared to the control littermates (Smn+/+; Smn2; SmnA7) were discernible before P7, but were not reliably predictive of genotype in individual mice (Figure 1A and 1B). SMN protein levels were markedly reduced in SMA mice at all time points as measured by Western blot (Figure 1C) and immunohistochemistry (Figure 1D). At P7 a failure in the righting reflex became apparent. Importantly, this was coincided with a drop in numbers of large motor neurons in the spinal cord (Figure 2A and 2B). No discernible differences in phenotype or pathology were present at P1, indicating that, in the SMnA7 mouse model of SMA, the disease develops in a motor system in which embryonic development has been relatively normal. By P13, mice appeared emaciated, were unable to right themselves, and showed signs of respiratory distress (Figure 1B). There was a corresponding loss of >30% of lower motor neurons from the ventral horn in SMA mice. At late-symptomatic stage (P13), a similar relative loss of motor neurons was evident across the entire length of the spinal cord, (Figure 2C), justifying the use of whole spinal cord for RNA analysis. However, absolute numbers differed depending on the region examined, reflecting the differential innervation of limb and trunk musculature by motor neurons originating in the cervical, thoracic or lumbar cord.

Global changes in the spinal cord transcriptome

To understand the scale of change in mRNA expression in the spinal cord, we first performed gene-level comparisons between genotypes at each time point, using the core probe sets on the array and GeneSpring software (Agilent Technologies, Santa Clara, CA, USA). When using an arbitrary p-value threshold of 0.05 and a fold-change threshold of 1.5 as cut-off for biological significance, the expression of 142 genes was increased or decreased in the spinal cord of SMA mice compared with their control littermates at late-symptomatic stage (P13), with a maximal fold-change of 3.9 (Table S1). Importantly, the degree of change between SMA and control mice was much smaller at the pre-symptomatic (P1) and early-symptomatic (P7) stages, with only 12 and 23 genes changed, respectively (Table 1 and Table 2; Figure 3A). This finding argues against a critical function of SMN in transcription regulation, but also shows that if widespread splicing changes occur in SMA, this does not lead to a pre-symptomatic systemic change in whole transcript level mRNA expression, which might be expected if mis-spliced transcripts are subject to nonsense-mediated decay.

An additional data analysis, which we refer to as the ENSG analysis, was performed in which probes were grouped into sets,
each corresponding to a gene in the Ensembl (version 49) annotation database [27]. Time point-specific differential expression between cases and controls was quantified by fitting a linear model on a gene-by-gene basis [28,29]. Of 21,911 genes examined, 693 genes exhibited case/control differences at P13, as opposed to 92 at P7, and 83 at P1 (Figure S1, Tables S2, S3, S4, see Text S1 for details of the linear model). This is in accordance with the parallel analysis described above.

We next examined changes between time points for each genotype. Overall, the number of genes differentially expressed between time points in control mice was higher by a factor of ten compared to the number of genes differentially expressed
between genotypes at individual time points (Figure 3B), suggesting that the immediate post-natal period is associated with major changes in spinal cord gene expression in normally maturing mice.

Exon-level analysis

A key aim of this study was to assess the amount of splicing variation in the spinal cord of an SMA mouse model compared to control mouse spinal cord at several time points during disease progression. Because exon-specific microarrays are relatively novel, and analysis methods have not been fully developed and validated, we used two complementary statistical approaches to investigate the number of differentially expressed exons. First we used an ANOVA test in GeneSpring (Agilent) to select exons which show a significant difference between exon-level and transcript-level signal and then calculated the splicing index (SI) [30], for these exons. The SI is the logarithm of the ratio of array probe set intensities (corresponding to expression levels of individual exons) to overall gene-level expression. So an SI value of 0 indicates equal expression of a particular exon in relation to the gene as a whole between genotypes i.e. no differential alternative splicing. Using a splicing index of $|SI| \geq 0.5$ as an arbitrary threshold we identified 252 potential alternative splicing events at the late-symptomatic stage (P13), but only 5 at the early symptomatic (P7) and 16 at the pre-symptomatic (P1) stage (Figure 4A). This initial analysis suggests that alternative splicing events are a consequence of disease progression in SMA, rather than the primary cause. Since the splicing index method is known to lead to inaccuracies if complex splicing patterns are present, such as splicing of multiple exons in one gene, and might thus underestimate the level of differential exon use present, we next performed an analysis comparing expression levels of individual exons between genotypes at each time point. The rationale for this is that each instance of differential splicing between genotypes will lead to at least one exon being differentially expressed. So the number of differentially expressed exons provides an upper bound for the number of differential splicing events. In this analysis, which we refer to as the ENSE analysis, probes were grouped into sets, each corresponding to an Ensembl exon [27]. Time point-specific differential expression between cases and controls was quantified by fitting a linear model on an exon-by-exon basis [28,29]. The p-value cut-off for significant differences between genotypes at the exon-level was chosen to balance sensitivity with a reasonable false discovery rate (FDR), as estimated by a permutation-based analysis (Text S1 and Table S5). At a p-value threshold of $1e^{-4}$, there were 812 significantly differentially expressed exons at P13, compared to 66 at P7, and 72 at P1 (Figure 4B, Tables S6, S7, S8); a total of 211,567 exons were...
Table 1. P7 gene-level changes SMA versus control, fold change >1.5, P < 0.05.

| Transcripts Cluster Id | Fold change | Regulation | Gene Title | Gene Symbol |
|------------------------|-------------|------------|------------|-------------|
| 6809524                | 3.5         | down       | survival motor neuron 1 | Smn1 |
| 6969997                | 2.6         | down       | hemoglobin, beta adult minor chain//hemoglobin, beta adult major chain | Hbb-b2//Hbb-b1 |
| 6805381                | 2.2         | up         | histone cluster 1, H1c | Hist1h1c |
| 6849595                | 2.2         | up         | cyclin-dependent kinase inhibitor 1A (P21) | Cdkn1a |
| 7018366                | 2.1         | up         | ectodysplasin A2 isoform receptor | Eda2r |
| 6961201                | 1.9         | up         | small nuclear ribonucleoprotein polypeptide A' | Snrpa1 |
| 6899760                | 1.9         | up         | thioredoxin interacting protein | Txnip |
| 6753306                | 1.7         | down       | olfactory receptor 420 | Ofr420 |
| 6842587                | 1.7         | down       | Chondrolectin | Chodl |
| 6790294                | 1.7         | up         | chemokine (C-C motif) ligand 3 | Ccl3 |
| 6764650                | 1.6         | up         | epoxide hydrolase 1, microsomal | Epha1 |
| 6754143                | 1.6         | up         | ribonuclease L (2', 5'-oligoisoadenylate synthetase-dependent) | Rnasel |
| 6926165                | 1.6         | up         | complement component 1, q subcomponent, beta polypeptide | C1qb |
| 6963128                | 1.6         | up         | olfactory receptor 635 | Ofr635 |
| 6873271                | 1.6         | down       | stearyl-Coenzyme A desaturase 1 | Scd1 |
| 6785114                | 1.6         | down       | RAB37, member of RAS oncogene family | Rab37 |
| 6926166                | 1.6         | up         | complement component 1, q subcomponent, C chain | C1qc |
| 7009748                | 1.6         | down       | diacylglycerol kinase kappa | Dgkk |
| 6788617                | 1.6         | down       | olfactory receptor 323 | Ofr323 |
| 6885616                | 1.5         | up         | RIKEN cDNA 1700007K13 gene | 1700007K13Rik |
| 6939671                | 1.5         | up         | transmembrane protease, serine 11d | Tmprss11d |
| 6870375                | 1.5         | up         | insulin 1 | Ins1 |
| 6783144                | 1.5         | down       | carbonic anhydrase 4 | Car4 |

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Expression analysis of Smn−/−;SMN2 spinal cord

While the results obtained from the SMNA7 mouse model afforded important insights into the dynamics of gene- and exon-level expression changes over time, to ensure that our findings were not restricted to this particular transgenic model of SMA, but applicable to SMA mouse models in general, we performed a similar analysis on the more severe but genetically less complicated Smn−/−;SMN2 mouse model of SMA [32]. The Smn−/−;SMN2 animals, which in complete absence of mouse Smn is rescued by the introduction of the human SMN2 transgene, have a maximum lifespan of 6 days. Previous studies showed that at P1, there are normal motor neuron numbers, whereas at P5, there is about a 35% loss compared to litter mates with normal mouse Smn−/−;SMN2 animals, in which complete absence of mouse Smn is rescued by the introduction of the human SMN2 transgene, have a maximum lifespan of 6 days. Early synaptic abnormalities are seen from P2 in this model [33], although neuromuscular junctions appear normal at P1, indicating normal pre-symptomatic development [34]. Exon-array analysis of spinal cord harvested from pre-symptomatic (P1) and late-symptomatic (P5) animals mirrored the principal findings in the SMNA7 mouse at both gene and exon level. When examining gene expression in Genespring using core probe sets, more changes were present at late-symptomatic stage than at the pre-symptomatic stage (3 genes up- or down-regulated at P1, 160 genes up- or down-regulated at P5 with p ≤ 0.05 and fold change > 1.5). Even more striking was the result of the splicing index analysis, which showed 27 potential alternative splicing events at P5, but none at P1 when choosing a splicing index cut-off of 0.3.
These results showed that our findings in the SMNΔ7 mouse model were not caused by a specific effect of the SMNΔ7 transgene but are likely to be a generalised phenomenon in SMA.

Validation of array findings by RT–PCR, western blotting, and immunohistochemistry

To validate the findings of our microarray experiments, we performed semi-quantitative and/or quantitative RT-PCR focusing on genes that showed at least one exon with differential expression at all time points (Cdkn1, Snrp1a, Chodl, Mccc2, Uspl1, ChAT, Figure S2). In addition, we performed qRT-PCR on spinal cord samples obtained from E16 embryos for some of the targets to examine whether changes were already present prenatally. All qRT-PCR results matched the changes at gene-level seen in the array experiments, underlining the robustness of the array findings.

Chodl, the gene encoding chondrolectin is a C-type lectin with unknown in vivo function. Interestingly, in situ hybridisation shows that this gene is highly expressed in anterior horn cells (Allen Brain Atlas http://mousespinal.brain-map.org) and might have important, if currently unknown, motor neuronal functions. We observed a progressive reduction in Chodl expression over time. Of note, the exon array data were indicative of differential expression of the 3′ end of Chodl, and validation by qRT-PCR using primers spanning both constitutive exons as well as two alternative 3′-terminal exons was in keeping with a preferential loss of the Chodl -001 isoform (ENSMUST23568) and relative sparing of Chodl-002 (ENSMUST69148) (Figure 5A and 5B). The relative reduction of Chodl expression appeared to be spinal cord specific and could not be demonstrated in either skeletal muscle or kidney. However, even these tissues showed a trend towards increased expression of the Chodl-002 isoform in SMA compared to control mice (Figure 5C). Immunohistochemistry for Chodl showed strong immunoreactivity of anterior horn cells, in keeping with the published in situ data. The reduced Chodl expression was at least partially due to loss of motor neurons (Figure S4).

Uspl1 (ubiquitin specific peptidase like 1), a gene encoding part of the ubiquitin-dependent protein degradation pathway was
cells with grey matter preference. In keeping with the only mild overall increase in Uspl1 expression at mRNA level (Figure S2F), no difference in immunoreactivity was evident between genotypes (Figure S4) and Western blotting showed no significant difference in the main protein isoform identified between genotypes.

Further interesting changes at all time points were detected including the whole-gene up-regulation of Snrpa1 (average 1.8 fold). Snrpa1 encodes one of the many protein components of the spliceosomal A complex [34], which is associated with the U2 snRNA. Western blotting showed a consistent small expression increase (Figure S4). While no other spliceosomal components were differentially expressed, the Snrpa1 change might reflect a compensatory response of the cell to Smn deficiency.

Another change included down-regulation of isoforms ENSEMBST00000091326 and ENSMUST00000022148 of Mccc2, the gene encoding the methylcrotonoyl-CoA carboxylase beta chain, a mitochondrial enzyme involved in amino acid metabolism (Figure S2D). Interestingly, the array data suggest that, while two Mccc2 isoforms were expressed at lower levels in SMA compared to controls, a third isoform (ENSMUST00000109383) was in fact up-regulated. This was confirmed by qRT-PCR and semi-quantitative PCR (Figure S3).

Pathway analysis

In the SMNΔ7 mouse model, the early postnatal days appeared particularly relevant to disease development. Our earlier finding of massive gene expression changes between time points in post-natal wild-type mice (Figure 3B) indicates that events relevant to disease in the SMNΔ7 mouse model coincide with transcriptome changes associated with normal post-natal development or maturation. To analyse which pathways were physiologically activated during this time, we first compared gene expression in control mice (Smn+/+; SMN2:SMNΔ7) between P1 and P7, and subsequently between P7 and P13. Using GO-Elite software, we identified pathways enriched with genes involved in spinal cord cell proliferation, axon development, oligodendrocyte development and myelination as significantly altered, reflecting physiological events during the rapid growth of the spinal cord. When the same analysis was performed for the SMA mice, a strikingly different pattern emerged. With the exception of two GO IDs pertaining to nervous system development, all physiologically activated pathways were absent in both the P1 v P7 and P7 v P13 analyses (Table 3). To investigate whether this dramatic change in gene expression pathways was mirrored by a difference in proliferating spinal cord cells, we performed Western blotting and immunohistochemistry for the proliferation marker PCNA. Our preliminary results indicate that there is indeed a reduction in the number of proliferating cells in the SMA spinal cord (Figure S5). At P13, genes relating to cellular responses to DNA damage became prominent in the SMA mice, which could not be explained by a significant amount of spinal cord gliosis (Figure S5). Of note, the gene with the highest-fold change between genotypes at P13 was Cdkn1a, a cyclin dependent kinase inhibitor activated by p53 in response to DNA damage. This analysis suggested that in the SMNΔ7 mouse model there is an inhibition or a failure of activation of the normal physiological pathways of post-natal spinal cord maturation.

Discussion

In this study we undertook a detailed assessment of transcriptional changes over time in the spinal cord of a commonly used severe mouse model of SMA, using time points correlated with key phenotypic and pathological changes. We identified alterations in

Figure 4. Exon-level changes are a late occurrence in SMN deficient mice. (A) Venn diagram depicting potential splicing events as evidenced by a Splicing Index [S]>0.5. 252 alternative splicing events are present at late-symtomatic SMA mice compared to controls, but only 5 at P7 and 16 at P1. (B) Venn diagram depicting exon-level changes of Ensembl exons between genotypes at each time point investigated. At P1, P7, and P13, each of 211,567 exons was tested for differential expression between SMA and control. 812 exons were associated with disease status at P13, compared to 72 exons at P1 and 66 exons at P7. doi:10.1371/journal.pgen.1000773.g004

found to be up-regulated in SMA at all time points. In addition, Uspl1 showed a consistent change in both splicing index and Ensembl exon-level analysis of differential splicing. Uspl1 exon 2 (ENSMUSE00000351955) is a cassette exon absent in transcripts Uspl1-006 (ENSMUST00000121416) and -007 (ENSMUST0000117878). The increased use of Uspl1 exon 2 in the SMA mice led to an isoform shift with relatively higher levels of exon 2 containing transcripts Uspl1-001-005 (Figure 6). Of note, this pattern was much more pronounced in muscle than in spinal cord, and less evident in kidney (Figure 6B). In spinal cord samples, the degree of isoform shift was more pronounced at symptomatic than at pre-symptomatic stages (Figure 6C). Immunohistochemistry for Uspl1 showed ubiquitous cytoplasmic staining in all spinal cord
a subset of genes involved in post-natal neurodevelopmental pathways in SMA, and showed that splicing alterations are only a late occurrence in disease.

Survival, weight development and motor phenotype of our SMN<sup>D7</sup> mouse colonies were similar to that described by others [26,35], with a change in outward appearance and development of motor deficits apparent at P7. Recent studies have identified morphological changes at the neuromuscular junction as early events in SMA [33,35] with neurofilament accumulation occurring as early as P5 in the SMN<sup>A7</sup> model. This structural change at the distal end of the motor neuron is closely followed in our study by a significant loss of large motor neurons at P7, indicating that although synaptic changes are the earliest identified feature of SMA it ultimately is a disease of the entire lower motor neuron. Of note, motor neuron loss was present to a similar degree across the entire spinal cord, in contrast to the previous finding of a rostral-caudal gradient with relative sparing of the lumbar region [35]. Motor neuron loss at P7 was reflected in the reduction at transcript level of choline acetyl transferase (ChAT), the key enzyme in motor neuronal synthesis of acetylcholine (Figure S2E). This finding, as well as that of reduced levels of Chodl mRNA, which seems to be highly expressed in anterior horn cells, shows that even though whole spinal cord was used for the array, important cell-type specific changes were detectable.

**Figure 5. Alternative splicing of Chodl.** (A) Graphical output of the exon-level analysis for Chodl. Each column, delineated by bold black lines, corresponds to the preprocessed data from a single Ensembl exon. The vertical axis displays log<sub>2</sub> expression for control (black) and SMA (red) animals, with each point corresponding to an individual animal. Each column is subdivided by vertical dashed lines into time points P1, P7, and P13 (left to right). Orange boxes mark those (exon, time point) combinations that exhibit significant differential expression between cases and controls. Expression of Chodl constitutive exons is reduced progressively from P1 to P13, but there is no difference between SMA and control for the alternative terminal exon ENSMUSE00000556896 indicating an isoform shift towards Chodl-002 (ENSMUST69148) in the SMA mice. Arrows indicate location of qRT–PCR primers for validation. (B) qRT–PCR results at P13 showing marked reduction in Chodl when measured using primers located in the constitutive exons 1–2 and the terminal exon of Chodl-001, while no significant difference of alternative exon ENSMUSE00000556896 exists between control and SMA (** = p < 1e-3). (C) The differential terminal exon usage is also evident in muscle and kidney in SMA mice, although overall transcript levels are not reduced.

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Figure 6. Alternative splicing of Usp11. (A) Graphical output of exon-level analysis for Usp11. Each column, delineated by bold black lines, corresponds to the preprocessed data from a single Ensembl exon. The vertical axis displays log2 expression for control (black) and SMA (red) animals, with each point corresponding to an individual animal. Each column is subdivided by vertical dashed lines into time points P1, P7, and P13 (left to right). Orange boxes mark those (exon, time point) combinations that exhibit significant differential expression between cases and controls. Expression of Usp11 is higher in SMA mice for all exons, but this difference is more pronounced for the first exon detected by the array, which corresponds to Usp11 exon 2 (ENSMUSE00000351955), reflecting a potential alternative splicing event. (B) Validation of the alternative splicing event by RT–PCR. Cartoon depicting primer position in Usp11 exon 1 and 3. RT–PCR was performed on four biological replicates at P13 showing exon 2 skipping in control mice, and increased exon 2 usage in SMA mice. The difference of the exon 2/–exon 2 ratio between genotypes is tissue specific and most pronounced in muscle and less obvious in kidney. (C) qRT–PCR results for exon 2 showing increased in Usp11 exon 2 expression in the SMA mice compared to control. The differential expression is more pronounced at symptomatic compared to pre-symptomatic stages.

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The key finding of this study is that alternative splicing events are a late occurrence in SMA, and are therefore unlikely to contribute to early disease pathogenesis. Zhang et al. [15] described widespread splicing abnormalities in several tissues including the spinal cord at end-stage disease in the same mouse model employed in this study and attributed this finding to dysfunction of the spliceosome secondary to SMN deficiency. Importantly, even though the time point of analysis is not absolutely identical to ours, there is considerable overlap between Zhang et al.’s data set obtained at P11 and our P13 data set, when raw data are analysed in the same way (Figures S6, S7; Tables S1, S9, S10, S11). However, it remains unclear from these data whether splicing abnormalities had preceded the onset of symptoms, as would be predicted from the crucial role of SMN in spliceosome assembly, particularly in embryonic and early postnatal development [13]. In addition, the presence of widespread splicing defects in organs other than the spinal cord is difficult to interpret in the light of apparent tissue specificity of the disease if splicing abnormalities are indeed thought to be relevant to the mechanism of motor neuron degeneration. To determine the degree of variation in splicing between SMA and control mice, we utilised the splicing index, but also examined changes at individual exons as a measure of the maximum number of alternative splicing events present. The absolute number of changes found in late-symptomatic mice was not large given the large number of exons examined (>200,000). When the same analysis was performed, comparing between genotypes at pre-symptomatic and early symptomatic time points, only very few exon-level changes were present. Importantly, we were able to confirm a similar pattern of exon expression changes in the more severe Smm−/−:SMN2 mouse model, corroborating our main finding in the SMA7 mice. Overall, our data indicate that the majority of splicing changes are not a direct consequence of SMN deficiency, but rather a consequence of disease progression, probably representing physiological isoform-shifts in response to cell stress. There is evidence that oxidative stress can induce shifts in alternative splicing, and that neurons may be more vulnerable to this process than other cells [36]. We would therefore argue that SMN deficiency to the degree observed in either the SMA7 or Smm−/−:SMN2 mouse, although associated with severe reduction in snRNP assembly capacity in vivo [14], does not lead to a systemic breakdown of splicing fidelity in vivo until the disease is well established.

While our results do not support the hypothesis that widespread, systematic splicing abnormalities cause SMA, we cannot rule out the possibility that splicing of one or several transcripts is critically affected by SMN deficiency, and that the few splicing changes observed early in our mice contribute to SMA pathogenesis, followed by a cascade of loss of splicing fidelity or secondary effects. In fact, at least one of the genes identified by our array (Usp11) is differentially spliced between SMA and control mice even at the embryonic phase, albeit to a lesser degree than at the symptomatic stages. Of note, the isoform shift observed in this particular gene is more pronounced in muscle than in spinal cord, and less marked in kidney, an organ not affected by SMA pathology.

Independent of whether or not splicing changes are ultimately responsible for SMA disease initiation, our study identified several pathways that might shed light on SMA pathogenesis and disease progression. Analysis of transcriptional changes between genotypes in this study took place on a background of large scale physiological changes between time points, reflecting rapid neuronal development in the early post-natal days. Analysis of the changes between time points identified several pathways related to normal neuronal development. Surprisingly, in the SMA mice the majority of physiological transcriptional changes seen in control mice were absent even between the early time points P1 and P7. This finding not only indicates that abnormal post-natal neuronal development might underlie early events in SMA but might explain general delayed growth and failure to thrive in the SMA mice.
In this study, we examined gene expression in the entire spinal cord and are unable to distinguish which cell types contribute most to expression changes, even though changes that plausibly originate from motor neurons, such as ChAT and Chodl, are clearly present. The neurodevelopmental pathways identified as altered in our study can be associated with other cell types, including oligodendrocytes and cells in the posterior spinal cord. While this is a preliminary finding, it clearly warrants further studies examining the role of non-motor neuronal cells in SMA.

Human autopsy cases indicate involvement of sensory neurons in the dorsal root ganglia, Clarke’s column and the thalamus in SMA [37–40], although the majority of studies were undertaken before the molecular diagnosis of SMA was available. More recent clinical studies showed that severe SMN-related SMA is associated with widespread neuronal degeneration, including sensory pathways [41]. Subtle sensory neuron abnormalities have also been detected in a severe mouse model of SMA [42]. To our knowledge, however, there is no study systematically investigating the role of non-motor neuronal cells in SMA spinal cord.

In conclusion, our data show that alternative splicing events predominantly occur late in SMA, while alterations of post-natal neurodevelopmental pathways precede overt symptom onset. Further studies should continue to focus on the role of SMN in the post-natal maturation and development of the neuromuscular system including spinal cord motor neurons.

### Materials and Methods

#### Mice

Transgenic Smn<sup>+/−</sup>;SMN2;SMNΔ7 [26] mice were maintained as heterozygous breeding pairs in standard animal facilities in Oxford. Homozygous Smn<sup>−/−</sup>; SMN2; SMNΔ7 mice reached the

| GOID | GO Name | GOID | GO Name |
|------|---------|------|---------|
| 10456 | cell proliferation in dorsal spinal cord | 33269 | internode region of axon |
| 5243 | gap junction channel activity | 43209 | myelin sheath |
| 33269 | internode region of axon | 43209 | myelin sheath |
| 14003 | oligodendrocyte development | |
| 5248 | voltage-gated sodium channel activity | |
| 22829 | wide pore channel activity | |

### Table 3. Comparison of between time point changes in control and SMA mice using GO Elite for >50% and 3 genes changed.

| Control P1 versus P7 | SMA P1 versus P7 |
|----------------------|------------------|
| GOID | GO Name | GOID | GO Name |
| 10456 | cell proliferation in dorsal spinal cord | 33269 | internode region of axon |
| 5243 | gap junction channel activity | 43209 | myelin sheath |
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disease end-point by post-natal day 13 (P13). Five mice of each genotype were sacrificed at age P1, P3, P5, P7, P9, P11 and P13 for motor neuron counts, and 4 mice of each genotype at P1, P7 and P13 for RNA and protein extraction.

Mice were genotyped using DNA extracted from tail-tips and standard PCR procedures.

For motor neuron counts, mice were terminally anaesthetised with i.p. pentobarbitone and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS.

For RNA and protein extraction, mice were killed by i.p. injection of pentobarbitone.

**Motor neuron counts and immunohistochemistry**

Spinal cords were dissected, post-fixed in 4% PFA for 2 hours, cryoprotected in 30% sucrose overnight, embedded in OCT medium and rapidly frozen in liquid nitrogen-cooled isopentane. 20 μm thick horizontal sections were cut on a cryostat and stained with 0.5% Cresyl violet with 0.04% acetic acid. A minimum of 30 non-adjacent sections covering the entire spinal cord segment of interest were scrutinised for large, polygonal, Nissl positive cells in the cervical, thoracic and lumbar segments using the cervical and lumbar enlargements as landmarks. For the time-course, only lumbar spinal cord was utilised.

6 μm thick paraffin section were cut and stained with standard Haematoxylin and Eosin. For immunohistochemistry, sections were incubated with the primary antibody (mouse anti-SMN antibody (1:320, BD Transduction lab), rabbit anti-Usp11 (1:600, Santa Cruz), mouse anti-Chodl (1:200, abcam), rabbit anti-PCNA (1:2500, abcam), goat anti-Chodl (1:400, Chemicon) for 40 minutes at room temperature or at 4°C overnight. Antibody binding was visualised using a Dako REAL EnVision kit according to manufacturer’s instructions.

Immunohistochemistry was carried out on several sections taken from two different paraffin blocks for two animals per genotype. Representative images are shown.

**RNA isolation and microarray**

Whole spinal cords were rapidly dissected and snap-frozen on dry ice. RNA was extracted using the Qiagen RNeasy Mini RNA extraction kit according to the manufacturer’s instructions.

The quality and RNA integrity was assessed on a BioAnalyzer; all samples had a RNA Integrity Number (RIN) ≥9 (Agilent Laboratories, US). 1 ug starting RNA was ribosome depleted using the Ribominus Human/Mouse Transcriptome Isolation kit (Invitrogen). Labelled sense ssDNA for hybridization was generated with the Affymetrix GeneChip WT sense target labelling and control reagents kit (Affymetrix, UK) according to the manufacturer’s instructions. Sense ssDNA was fragmented and the distribution of fragment lengths was measured on the BioAnalyzer. The fragmented ssDNA was labelled and hybridized to the Affymetrix GeneChip Mouse Exon 1.0 ST Array (Affymetrix).

Chips were processed on an Affymetrix GeneChip Fluidics Station 450 and Scanner 3000.

**Microarray gene expression and pathway analysis**

For the gene-level analysis, core probe sets which map to the same transcript cluster were grouped together and RMA (Robust multi-array analysis) [44] normalised in GeneSpring GX10.1.02. Differentially expressed genes were identified using an unpaired t-test; selecting genes with 1) a p-value less than or equal to an arbitrary threshold of 0.05 and 2) a fold change difference between genotypes ≥1.5. The selected genes were sorted according to gene ontology using GenMAPP's GO-Elite (http://www.genmapp.org/go_elite/go_elite.html). Only MAPPfinder ontologies with ≥3 genes changing and a permuted p-value of ≤0.05 were reported.

**Splicing index**

At the exon level, core probe sets were PLIER (Probe Logarithmic Intensity Error) normalised in GeneSpring GX 10.1.02 (Agilent). Transcript probe sets that had detection above background (DABG) p-value≤0.05 in both SMA and control groups were retained. An ANOVA test was used to identify significant differences between exon-level signal and transcript level signal. As recommended in the Affymetrix White Paper [44,45], exon level probe sets exhibiting exon-to-transcript intensity ratios >5 were excluded from the ANOVA (where log2(exon-to-transcript ratio) = log2(exon expression) – log2(transcript expression)). This filter removed probes with high background and cross-hybridisation potential. The p-value threshold for the ANOVA was selected to control for a false discovery rate of 0.05 using the Benjamini-Hochberg multiple testing procedure [46]. For exons selected on the basis of the ANOVA, the splicing index, SI = log2(exon-to-transcript expression ratio), was calculated and used as a measure of differential splicing between genotypes. See [43,47] for further details. A significantly differentially spliced exon was defined to be one having both an FDR-controlled ANOVA p-value≤0.05 and |SI| ≥0.5 (on the log scale, corresponding to a fold change up or down of approximately 1.4 in the absolute exon/transcript ratio between genotypes).

**Ensembl exon and Ensembl gene expression analysis**

CEL files were preprocessed using RMA without background correction (see Text S1). Publicly available custom chip-definition files (CDFs) (http://brainarray.mbbi.med.umich.edu/Brainarray/Database/CustomCDF/CDF_download.asp) were used to group probes into sets. Parallel analyses, based on two different CDFs were performed. The first CDF, referred to as ENSE, defines a probe set for each Ensembl exon. The second, ENSG, defines a probe set for each Ensembl gene [27].

There were 211,567 and 21,911 probe sets for the ENSE and ENSG analyses respectively. At each probe set, a linear model was fitted using the limma package (version 2.16.5), to quantify evidence of genotype differences within each time point (see Text S1 for details).

**False discovery rate analysis**

A permutation-based analysis was conducted to estimate the FDR at a variety of p-value thresholds (see Text S1 and Table S5). The p-value thresholds selected were 1e-4 for ENSE, and 1e-3 for ENSG, as these thresholds controlled the FDR at a reasonably low level.

Statistical analyses were performed using R [28], version 2.8.1.
Protein extraction

Snap frozen whole spinal cords were homogenised in RIPA lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% (v/v) SDS, 1% (v/v) sodium deoxycholate, 1% (v/v) TX-100) and 1% (v/v) protease inhibitors by sonication at 50% output for 15 sec. Homogenates were chilled on ice for 20 min and clarified by centrifugation at 15,800 g for 20 min at 4°C.

Immunoblotting

Protein samples (50 µg/well) were electrophoresed through 12% SDS polyacrylamide gels and transferred to 0.2 µm nitrocellulose membranes (Millipore). Membranes were blocked with 5% (w/v) milk powder in TBS-T, pH 8.0, for 1 hr and incubated with the primary antibody (mouse anti-SMN (1:1,000, BD Transduction labs), mouse anti-actin (1:1,000, Abcam), rabbit anti-USPL1 (1:1,000, abcam), rabbit anti-SNRPA1 (1:1000, abcam), goat anti-CHAT (1:500, Chemicon), rabbit anti-USPL1 (1:500, Santa Cruz), rabbit anti-PCNA (1:200, abcam) in 3% (w/v) BSA in TBST overnight at 4°C. Membranes were incubated with the primary antibody (mouse anti-SMN (1:1,000, Abcam), rabbit anti-SNRPA1 (1:1000, abcam), goat anti-CHAT (1:500, Chemicon), rabbit anti-USPL1 (1:500, Santa Cruz), rabbit anti-PCNA (1:200, abcam) in 3% (w/v) BSA in TBST overnight at 4°C. Blots were probed with HRP-conjugated antibodies (1:10,000, Amersham) and developed using ECL reagents (Amersham).

Western blots were repeated three times on biological replicates, and representative blots are shown.

RT–PCR and qRT–PCR

RNA was reverse transcribed into cDNA using random hexamer primers (Invitrogen) and Expand Reverse Transcriptase (Roche) under standard conditions.

RT–PCR was performed using Taq DNA Polymerase (Sigma) or Expand High Fidelity PCR system (Roche). Real-time PCR was performed using Fast SYBR Green chemistry (Applied Biosystems) and a StepOnePlus Real-time PCR machine (Applied Biosystems). Real-time PCR Primers were designed with Primer Express software (Applied Biosystems). Primer concentrations were optimised to yield low Ct values and minimal primer dimer formation (commonly 300 nM for both forward and reverse primers). GAPDH was used as the endogenous control, as there was no differential expression between genotype in the array and in qRT–PCR experiments. All primer pairs were tested to have similar amplification efficiency to GAPDH when tested on serial cDNA dilutions over 4 log. Fold change was calculated using standard ΔΔCt calculations. The average fold change per time point was calculated from four biological replicates at each time point, and an unpaired two-tailed t-test was used to test for significance (p≤0.05, **= p≤0.01, *** = p≤1e-3).

Supporting Information

Figure S1 Gene level expression changes of Ensembl Genes. Venn diagram showing the number of differentially expressed genes at different time points with p≤1e-3.

Figure S2 Array validation by qRT–PCR. Quantitative RT–PCR was carried out for all time points on the gene displaying the highest fold-change in late-symptomatic mice, but no change at the pre-symptomatic stage (A: Cdkn1a) as well as several targets found to be differentially expressed at several time points in the exon array ENSE analysis (B–F: Chodl, Snrpl1, Mccc2, ChiAT, Uspl1). Genes showing differential expression at P1 were also examined at embryonic stage E16. Expression is shown relative to control animals. GAPDH was used as the endogenous control. All qRT–PCR results are in agreement with the expression change predicted by the array.

Error bars show the standard deviation of the mean for both 4 control and 4 SMA animals per time point. An unpaired t-test was performed between genotypes to test for significance (* = p≤0.05, ** = p≤0.01, *** = p≤1e-3).

Found at: doi:10.1371/journal.pgen.1000773.s002 (0.48 MB TIF)

Figure S3 Differential expression of Mccc2 isoforms. (A) Graphical output of exon array for Mccc2 (analogous to the graphical outputs of exon array data in the main manuscript). (B) At P13, qRT–PCR across exons present in isoforms Mccc2-201 (ENSEMUST00000091326) and Mccc2-202 (ENSEMU- ST00000022148) shows reduced expression in SMA compared to control (** p≤1e-3, unpaired t-test). (C) While the reduced expression level of Mccc2-201 and Mccc2-202 is not apparent on semi-quantitative RT–PCR, the Mccc2-203 isoform ENSEMU-ST00000109383 shows increased expression in SMA.

Found at: doi:10.1371/journal.pgen.1000773.s003 (0.83 MB TIF)

Figure S4 Validation of array findings at protein level. (A) Immunohistochemistry for Chodl on spinal cord sections of P13 control (A,C) and SMA (B,D) mice shows reduced Chodl immunoreactivity in the ventral horn of SMA mice, but no complete loss of Chodl from remaining anterior horn cells. Similar results are obtained for Chat in control (E,G) and SMA (F,H) mice. Both Chodl and Chat preferentially stain large anterior horn cells. Staining for Uspl1 (I,J) shows ubiquitous cytoplasmic Uspl1 expression with preference of the grey matter. (B) Chodl immunohistochemistry on adult human spinal cord shows very specific labelling of motor neurons in the ventral horn, supporting the importance of Chodl for motor neurons. (C) Western blotting of P13 spinal cord lysates shows reduced Smn and Chat protein levels, minimal increase of Snrpal and no overall difference in Uspl1. The Uspl1 antibody detected multiple bands in keeping with several known Uspl1 isoforms. MW, molecular weight in kDa. Scale bars 100 µm.

Found at: doi:10.1371/journal.pgen.1000773.s004 (5.48 MB TIF)

Figure S5 Markers of spinal cord proliferation and gliosis. (A) GFAP immunohistochemistry of control (A,C) and SMA (B,D) mice shows no significant difference in spinal cord gliosis at P13. (B) Western blotting for the cell proliferation marker PCNA (Proliferating Cell Nuclear Antigen antibody) shows a decrease in SMA. (C) The specificity of the antibody is shown by staining of rostral migratory stream cells [A] no primary antibody, [B] rabbit anti-PCNA 1:2500] in mouse brain. (D) The entral canal ependymal zone contains several PCNA positive cells in control (A,C,E), but not in SMA mice (B,D,F). GFAP, glial fibrillary acidic protein.

Found at: doi:10.1371/journal.pgen.1000773.s005 (6.13 MB TIF)

Figure S6 Effect of RMA background correction. RMA background correction applies a smooth, monotonic transformation from raw probe intensities to corrected probe intensities (this figure displays this transformation for a single exon array). The function is linear for medium-to-high intensities, but tends to stretch out the low-intensity range (the figure is annotated with a two-fold interval that is mapped to a ten-fold interval).

Found at: doi:10.1371/journal.pgen.1000773.s006 (0.25 MB TIF)

Figure S7 Comparison of P13 data set and Zhang et al data set. This figure (ENSE (A), ENSG (B)) compares the log2(casc/control fold change) across studies. Only probe sets that are significantly differentially expressed in at least one study are included. There is clearly a degree of concordance between the two studies at these probe sets. In particular, the directionality of differential expression is extremely consistent across studies (Table S2).
Alternative Splicing Events Occur Late in SMA

Table S1  P13 gene level changes SMA vs control, fold change >1.5, P≤0.05. Found at: doi:10.1371/journal.pgen.1000773.s007 (0.63 MB TIF)

Table S2  ENSG analysis P1. Differentially expressed genes at P1 under the ENSG annotation. ENSG data: “+” indicates over-expression of cases relative to controls. Found at: doi:10.1371/journal.pgen.1000773.s008 (0.22 MB DOC)

Table S3  ENSG analysis P1. Differentially expressed genes at P1 under the ENSG annotation. Found at: doi:10.1371/journal.pgen.1000773.s009 (0.01 MB CSV)

Table S4  ENSG analysis P7. Differentially expressed genes at P7 under the ENSG annotation. Found at: doi:10.1371/journal.pgen.1000773.s010 (0.01 MB CSV)

Table S5  ENSG analysis P13. Differentially expressed genes at P13 under the ENSG annotation. Found at: doi:10.1371/journal.pgen.1000773.s011 (0.01 MB CSV)

Table S6  ENSE analysis P1. Differentially expressed exons at P1. Found at: doi:10.1371/journal.pgen.1000773.s012 (0.06 MB CSV)

Table S7  ENSE analysis P7. Differentially expressed exons at P7. Found at: doi:10.1371/journal.pgen.1000773.s013 (0.01 MB CSV)

Table S8  ENSE analysis P13. Differentially expressed exons at P13.

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Table S9  ENSG comparison P13 vs Zhang et al. Probe sets that are case/control differentially expressed (p≤1e-3) under the ENSG annotation in at least one of (a) the P13 data, (b) Zhang’s spinal cord data. Along with the exon/gene IDs and the number of exons assayed in each gene, the tables include p-values, log2(fold change), and signed fold change. Found at: doi:10.1371/journal.pgen.1000773.s016 (0.14 MB CSV)

Table S10  ENSE comparison P13 vs Zhang et al. Probe sets that are case/control differentially expressed (p≤1e-4) under the ENSG annotation in at least one of (a) the P13 data, (b) Zhang’s spinal cord data. Along with the exon/gene IDs and the number of exons assayed in each gene, the tables include p-values, log2(fold change), and signed fold change. Found at: doi:10.1371/journal.pgen.1000773.s017 (0.25 MB CSV)

Text S1  Statistical analysis of microarray findings; comparison of the P13 dataset with Zhang et al; primer sequences. Found at: doi:10.1371/journal.pgen.1000773.s018 (0.09 MB PDF)

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Author Contributions
Conceived and designed the experiments: DB KED KT. Performed the experiments: DB SL LMM THG OA. Analyzed the data: DB SL GN JLD OA. Contributed reagents/materials/analysis tools: SL GN NJP LMM OA. Statistical analysis of microarray findings; comparison of the P13 dataset with Zhang et al; primer sequences: THG OA. Wrote the paper: DB SL GN LMM THG KED KT.
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