A link between agrin signalling and Ca\textsubscript{v}3.2 at the neuromuscular junction in spinal muscular atrophy

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SMN protein deficiency causes motoneuron disease spinal muscular atrophy (SMA). SMN-based therapies improve patient motor symptoms to variable degrees. An early hallmark of SMA is the perturbation of the neuromuscular junction (NMJ), a synapse between a motoneuron and muscle cell. NMJ formation depends on acetylcholine receptor (AChR) clustering triggered by agrin and its co-receptors lipoprotein receptor-related protein 4 (LRP4) and transmembrane muscle-specific kinase (MuSK) signalling pathway. We have previously shown that flunarizine improves NMJs in SMA model mice, but the mechanisms remain elusive. We show here that flunarizine promotes AChR clustering in cell-autonomous, dose- and agrin-dependent manners in C2C12 myotubes. This is associated with an increase in protein levels of LRP4, integrin-beta-1 and alpha-dystroglycan, three agrin co-receptors. Furthermore, flunarizine enhances MuSK interaction with integrin-beta-1 and phosphotyrosines. Moreover, the drug acts on the expression and splicing of \textit{Agrn} and \textit{Cacna1h} genes in a muscle-specific manner. We reveal that the \textit{Cacna1h} encoded protein Cav3.2 closely associates in vitro with the agrin co-receptor LRP4. In vivo, it is enriched nearby NMJs during neonatal development and the drug increases this immunolabelling in SMA muscles. Thus, flunarizine modulates key players of the NMJ and identifies Ca\textsubscript{v}3.2 as a new protein involved in the NMJ biology.

Spinal muscular atrophy (SMA) is characterized by the degeneration of spinal motoneurons and muscle atrophy. SMA is caused by mutations of the survival motor neuron 1 (SMN1) gene leading to the reduction of SMN protein levels\textsuperscript{1,2}. This protein is a ubiquitously expressed DNA/RNA-binding protein involved in the regulation of gene expression, including transcription, splicing, RNA transport and translation, the most characterized function being its role in the U-rich small nuclear ribonucleoproteins (snRNPs) biogenesis\textsuperscript{3–5}. In humans, the presence of a copy gene SMN2 provides less fully functional protein due to an alternative splicing of the last coding exon 7\textsubscript{6}. Accordingly, milder SMA phenotypes are observed when the number of copies of SMN2 gene increases\textsuperscript{7,8}. The approved SMA therapies are dedicated to increase SMN protein levels using either SMN gene therapy or by targeting SMN2 pre-mRNA with an antisense oligonucleotide (ASO) or a small-molecule. Despite these tremendous scientific and clinical advances, several patients are unable to take those medications, others poorly respond and sometimes, adverse effects are seen\textsuperscript{9–13}. Thus, a better understanding of the mechanisms underlying disease should contribute to the emergence of better therapies that are of clinical importance.

Defects of the neuromuscular junction (NMJ) have been observed in the pathogenesis of SMA before motoneuron loss consistent with a “dying-back” phenotype\textsuperscript{14–16}. Indeed, delayed maturation, smaller endplate area and endplate denervation are detected in SMA model mice\textsuperscript{17–19}. A hallmark of the NMJs is the clustering of muscular acetylcholine receptors (AChRs) on the postsynaptic muscle membrane mediated by the agrin secreted from the motoneurons\textsuperscript{20}. Agrin binds the receptor LRP4 and then, the agrin-LRP4 complex binds and activates the transmembrane MuSK promoting AChR clustering through adaptors such as docking protein 7 (Dok7)\textsuperscript{21,22}. Moreover, mice lacking functional \textit{Agrn}, \textit{Lrp4} or \textit{Musk} gene die perinatally.

The \textit{Agrn} gene produces multiple isoforms in various tissues using two promoters and alternative splicing of exons encoding domains of the C-terminus, such as the Y and Z sites\textsuperscript{23–25}. The alternative first exons encode either the secretory (NtA) or the transmembrane (Tm) N-termini, the latter being mainly expressed in neurons whereas NtA is also expressed in non-neural cells such as muscles\textsuperscript{26}. The neural Z’ agrin isoforms greatly clusters the AChRs compared to the muscle Z- agrin\textsuperscript{23}. As components of the extracellular matrix (ECM), agrins also bind to laminin, heparin, α-dystroglycan and β1 integrins\textsuperscript{27,28}. Thus, agrin is also involved in a variety of functions: it...
Flunarizine promotes the AChR clustering in the murine C2C12 myotubes. (A) Schematic representation of the C2C12 muscle cell cultures and treatment. (B) Fluorescence imaging of the AChR clusters with α-bungarotoxin (α-BTX) on DMSO- and flunarizine (FZ)-treated myotubes. (C) A flunarizine dose–response curve of AChR clustering (3 ≤ n ≤ 5 for each concentration of 100, 250, 500, 1000, 4000 and 10,000 nM, except 2 for 10 μM). (D) Concentration–response curve of the effects of flunarizine on Cav3.2 channels. For each point, mean current density was normalized to mean current density of the vehicle group (7 < n < 24 for each concentration, error bars are SEM). (E) Both the size and number of AChR clusters increased with the drug. The columns represent the number of clusters for the same number of myotubes per condition were analysed using ImageJ from 3 independent experiments (345 myotubes per condition). (F) Expression of endogenous proteins in total protein extracts of C2C12 myoblasts in proliferation medium (PM) and DMSO- and FZ-treated myotubes in differentiation medium during 7 days (Diff7). Cells were harvested after an overnight DMSO and FZ treatment. The PVDF membrane was immunoblotted with the indicated antibodies with or without a stripping step. α-tubulin serves as a loading control. A 50% increase of GAPDH protein levels is detected relative to the tubulin loading control with the drug. Uncropped images are in supplemental Fig. 3. (G) The columns represent fold–changes of protein expression in FZ-treated myotubes compared to the DMSO treatment (1 μg/ml). (H) Effects of blocking antibodies to agrin (anti-agrin), integrin β1 (anti-ITGB1) and α-dystroglycan (α-Dg1) on the AChR clustering in the presence of flunarizine (FZ) compared to negative control IgG. (I) Columns represent the number of clusters (size > 5 μm) from 40 randomly selected fields of each condition per experiment. Error bars represent SD from the mean values of 3 independent experiments. Diluted DMSO is used in control conditions because flunarizine is insoluble in aqueous solution. Khi-2 test, *, P < 0.05; **, P < 0.01; ***, P < 0.001. Scale bar, 30 μm.

maintains the NMJ29, promotes heart regeneration30, inhibits neurite elongation31–33, regulates synaptogenesis in the central nervous system34 and coordinates a crosstalk between LRP4/MuSK and integrin–β1 pathways in liver cancer35,36.

The role of agrin/LRP4/MuSK/Dok7 signalling pathway in SMA disease was first underlined by the observation that agrin Z⁺ exon is mis-spliced in spinal motoneurons of SMA model mice37, that can be corrected by the co-expression of AAV-9 U7-specific Lsm10 and Lsm11 proteins38. Modulation of agrin signalling by either the overexpression of a transgenic Z⁺ agrin, injection of a therapeutic agrin or AAV-Dok7 delivery mitigates the phenotype of SMA mice39–41. Also, MuSK agonist antibody reduces the neuromuscular defects in SMA mice42. Thus, improving the NMJ function through agrin signalling has therapeutic effects on the pathogenesis in SMA model mice. Yet, the expression of the Agrn exons other than the Z⁺ exons remains largely unexplored in SMA mice and after treatments.

The implication of NMJs in SMA disease was further illustrated when we reported that flunarizine increases the endplate area and maturation of NMJs and improves disease phenotype in SMA mice, but that independently of the levels of snRNAs and of Agrn Z⁺ exons43. The exact mode of action on the NMJ remains unknown. Flunarizine was previously used as a T-type calcium channel blocker44 and was identified in a chemical screen as a splicing regulator45. Both calcium levels and RNA metabolism are altered in SMA models46–51. Moreover, ASO treatment of SMA mice mitigates the splicing defects of U12-dependent introns of several genes including Cacna1h encoding the T-type calcium channel Ca,3.245.

We then envisaged an action of flunarizine on the expression and splicing of Agrn and Cacna1h genes in skeletal muscles. To address this question, we studied how flunarizine increases the NMJ size in control and SMA model mice. Since the drug effects are muscle-dependent and SMN-independent43, we assessed the effects of flunarizine on cultured murine C2C12 myotubes. We report that the drug stimulates in vitro the AChR clustering, an early event in the NMJ formation. This effect has a concentration dependence similar to that of the inhibition of Ca,3.2 current suggesting it requires channel block. Moreover, we reveal in vitro and in vivo that the expression and splicing of Agrn and Cacna1h genes appear to be co-regulated by flunarizine. We also show that the agrin co-receptor LRP4 associates with Ca,3.2 that we find localized nearby NMJs in murine neonatal period. Importantly, the Ca,3.2 immunolabelling in SMA muscles is increased by flunarizine. Hence, we propose flunarizine effects to associate Ca,3.2 with the formation and maturation of perinatal NMJs.

Results

Flunarizine enhances the AChR clustering in C2C12 myotubes. We previously showed that flunarizine enhances post-synaptic NMJs independently of SMN protein levels in control and SMA model mice43. This implies that muscles might respond directly to flunarizine. To test this idea, we assessed whether flunarizine stimulates in vitro the formation of AChR clusters in cultured C2C12 mouse myoblasts fused to form myotubes (Fig. 1A). AChR clusters are visualized using a fluorescent α-bungarotoxin (α-BTX). Flunarizine (4 μM) significantly increased 3–to fourfold the number and size of clusters (Fig. 1B,E). Moreover, flunarizine-induced clustering was dose-dependent (Fig. 1C). The half-maximal concentration (EC50) was 1.3 μM and 4 μM gave maximal effects on clustering. We also assessed flunarizine on the T-type calcium channel Ca,3.2 in stably expressing HEK293 cells (Fig. 1D). Electrophysiological recordings revealed that a similar drug concentration–dependence is found for flunarizine on Ca,3.2 channel activity (IC₅₀ = 3.44 μM). Of note, the time course of flunarizine inhibition illustrates that a maximum inhibition by 10 μM flunarizine is reached after a 5 min incubation period (supplemental Fig. 7). Thus, flunarizine stimulates AChR clustering most probably through a direct action on the Cav3.2 channel, suggesting a direct role of muscle cells in the beneficial effects of the drug in SMA model mice.
Flunarizine modulates NMJ-related protein levels in C2C12 myotubes. To dissect the flunarizine mechanisms inducing AChR clustering, we examined the protein levels of key NMJ molecules focusing on AChRα subunit, Lrp4, MuSK, dystroglycans (dg), Ca2+/calmodulin-dependent protein kinase II (CaMKII), integrin β1 and α11 in C2C12 myotubes (Fig. 1FG). No effect of flunarizine (4 μM) was detected on the protein levels of AChRα, MuSK and β-dystroglycan whereas 30, 50, 60 and 70% increases were observed for Lrp4, β1 and α11 integrins and α-dystroglycan epitope respectively. We also considered whether dynamin2 (Dyn2) overexpression induces AChR clustering and regulates NMJ development. An increase of Dyn2 and GAPDH pro-
Flunarizine influences Agrn gene expression in C2C12 myotubes and skeletal muscles. Results presented so far indicated that flunarizine potentiates the AChR clustering activity of C2C12-derived agrin. We then evaluated whether the drug might impact on muscle Agrn gene expression and its spliced isoforms. Agrins are expressed from two different transcriptional start sites giving rise to two N-termini, secreted NtA or transmembrane TM, followed by a common sequence and the alternative Y and Z exons encoding the C-terminal region which contains the binding sites for integrin-β1 and LRP4, respectively 28 (Fig. 2A). Knowing that the Z-site greatly enhances the AChR clustering 46, it was possible that flunarizine could promote the inclusion of Z exons. In fact, we illustrate that they are neither present nor induced by the drug in C2C12 myotubes, ruling out a possible involvement of CaMKII, which also regulates the AChR expression and clustering 54,55. Thus, flunarizine may promote agrin signalling since Lrp4/MuSK, β1 integrins and α-dystroglycan are all agrin receptors involved in NMJ biology 29

The only source of agrin in our experiments comes from C2C12 myotubes. We then asked whether antibodies that block agrin, integrin-β1 or α-dystroglycan affected the flunarizine-induced AChR clustering. The myotubes were treated with the drug and anti-α-grin mouse monoclonal antibody Mab5204 49 or the anti-integrin-β1 mouse monoclonal antibody Mab1965 57 or the anti-α-dystroglycan mouse monoclonal antibody IIIH6C4 59 or a negative control mouse antibody (Fig. 1H,I). Under our conditions, Mab5204 and Mab1965 antibodies markedly inhibited AChR clustering, but not IIIH6C4 and control antibodies. Besides, α-dystroglycan localized to all AChR clusters in flunarizine-treated myotubes (Supplemental Fig. 4), further supporting a role in cluster stabilization rather than formation 59. Thus, flunarizine-induced AChR clustering depends on agrin and integrin-β1 in vitro.

Flunarizine levels was shown with flunarizine. No drug effects were observed on CaMKII protein levels or phosphorylation, ruling out a possible involvement of CaMKII, which also regulates the AChR expression and clustering 46,55. SMN and Unrip (another SMN-complex component) protein levels were unchanged, as expected 56. Thus, flunarizine may promote agrin signalling since Lrp4/MuSK, β1 integrins and α-dystroglycan are all agrin receptors involved in NMJ biology 29.

To uncover whether other Agrn isoforms might be produced with flunarizine, we designed RT-qPCR primers for Agrn exons, namely NtA, Tm, ex5-6 (included in all isoforms), ex29-30 (3' region of all isoforms) and the inclusion of the Y-insert (Supplemental Table 2). C2C12 differentiation was associated with a decrease in total Agrn mRNA levels (ex5-6) and increased inclusion of the Y-insert (Fig. 2C). The NtA levels were unchanged upon differentiation but Tm levels were markedly reduced. Flunarizine reduced by ≈25% the NtA exon levels in C2C12 myotubes with the exon/ex5-6 ratios being unchanged (Fig. 2D). Furthermore, the proportion of NtA versus Tm showed that there is more NtA than Tm isoforms (as expected) and that an increase of NtA over Tm isoforms is observed upon differentiation as well as a reduction by flunarizine (Fig. 2E). Thus, we found that flunarizine-induced AChR clustering correlates with Y/Z Agrn isoforms in C2C12 myotubes.

To investigate Agrn expression in skeletal muscles, we explored Agrn exon levels in three hindlimb muscles, namely soleus, plantaris and tibialis from vehicle (V)- and flunarizine (FZ)-treated control (heterozygotes) and SMA model mice at post-natal day 10 (P10). These muscles differ in term of maturation, as indicated by the proportion of embryonic and neonatal myosin isoforms, and by the composition in type I and type II fibers 43. The maturation of two extensor muscles (soleus and plantaris) is altered in SMA model mice whereas the flexor tibialis is less affected as indicated by the expression of neonatal myosin heavy chain 17,43. We chose a time point to uncover whether other Agrn isoforms might be produced with flunarizine, designed RT-qPCR primers for Agrn exons, namely NtA, Tm, ex5-6 (included in all isoforms), ex29-30 (3' region of all isoforms) and the inclusion of the Y-insert (Supplemental Table 2). C2C12 differentiation was associated with a decrease in total Agrn mRNA levels (ex5-6) and increased inclusion of the Y-insert (Fig. 2C). The NtA levels were unchanged upon differentiation but Tm levels were markedly reduced. Flunarizine reduced by ≈25% the NtA exon levels in C2C12 myotubes with the exon/ex5-6 ratios being unchanged (Fig. 2D). Furthermore, the proportion of NtA versus Tm showed that there is more NtA than Tm isoforms (as expected) and that an increase of NtA over Tm isoforms is observed upon differentiation as well as a reduction by flunarizine (Fig. 2E). Thus, we found that flunarizine-induced AChR clustering correlates with Y/Z Agrn isoforms in C2C12 myotubes.
sites are not similarly sensitive to the drug (Fig. 2L–N). Also, the Y exon/ex5–6 ratio showed with flunarizine a value similar to controls in SMA plantaris and tibialis, but not in soleus. Thus, expression of Agrn isoforms is altered in SMA mice in a muscle-specific manner that flunarizine modulates.

**Flunarizine changes Cacna1h gene expression in C2C12 myotubes and skeletal muscles.** The lack of full correlation between the expression levels of Agrn isoforms (Fig. 2) and flunarizine action on NMJ size suggests that additional events contribute to the drug effects. Because flunarizine is classified as a blocker
of T-type calcium channels Ca₃.1, Ca₃.2 and Ca₃.3 encoded by Cacna1g, Cacna1h and Cacna1i genes, respectively⁴⁴, we then investigated whether the drug could modulate Cacna1h expression. Of note, Cacna1g and Cacna1i transcripts were below our revelation limits and are thus not detectable. Thus, primers were designed and validated for the analysis of expression and splicing of Cacna1h gene: ex2-3 (exons flanking an U12-dependent intron), ex15-16, ex25 and ex29-30 exons of Cacna1h gene is performed in C2C12 myotubes compared to myoblasts in proliferation medium (PM). (C) The expression ratios between exons and ex15-16 RNA levels, ex15-16 being present in all Cacna1h mRNAs. Total RNA is prepared from C2C12 myoblasts grown in proliferating medium (PM) or after 7 days in differentiation medium (Diff7) with either DMSO or flunarizine (FZ) for the last 16 h. (D) Immunoblot analysis of the Cav3.2 encoded protein Cav3.2 confirms a significant 20% reduction of the protein levels in total protein extracts of C2C12 myotubes by flunarizine (n = 3). Uncropped images are found in supplemental Fig. 3. (E–G) RNA levels of Cacna1h exons are determined at P10 in the soleus, plantaris and tibialis, respectively. (H–J) Expression ratios between exons and ex5-6 RNA levels are calculated for the soleus, plantaris and tibialis, respectively. Error bars represent SD from the mean values of triplicates from 3 independent experiments with C2C12 cells and 3 mice per group. NS, not significant (P > 0.05), *P < 0.05, ** < 0.01, ***P < 0.001. Student’s t-test.

Figure 3. Effects of flunarizine on the expression and splicing profile of Cacna1h gene in C2C12 myotubes and skeletal muscles of SMA model mice. (A) Schematic representation of the U12-intron flanked by exon 2 and 3 (ex2-3) and the alternative spliced exon 25 of Cacna1h gene is shown. (B) RT-qPCR analysis of the RNA levels of ex2-3, ex15-16, ex25 and ex29-30 exons of Cacna1h gene is performed in C2C12 myotubes compared to myoblasts in proliferation medium (PM). (C) The expression ratios between exons and ex15-16 RNA levels, ex15-16 being present in all Cacna1h mRNAs. Total RNA is prepared from C2C12 myoblasts grown in proliferating medium (PM) or after 7 days in differentiation medium (Diff7) with either DMSO or flunarizine (FZ) for the last 16 h. (D) Immunoblot analysis of the Cav3.2 encoded protein Cav3.2 confirms a significant 20% reduction of the protein levels in total protein extracts of C2C12 myotubes by flunarizine (n = 3). Uncropped images are found in supplemental Fig. 3. (E–G) RNA levels of Cacna1h exons are determined at P10 in the soleus, plantaris and tibialis, respectively. (H–J) Expression ratios between exons and ex5-6 RNA levels are calculated for the soleus, plantaris and tibialis, respectively. Error bars represent SD from the mean values of triplicates from 3 independent experiments with C2C12 cells and 3 mice per group. NS, not significant (P > 0.05), *P < 0.05, ** < 0.01, ***P < 0.001. Student’s t-test.
snRNA levels were unchanged with the drug (Supplemental Fig. 1), our data associate minor U12 spliceosomes as potential targets of the drug in C2C12 myotubes.

Next, we measured *Cacna1h* gene expression in skeletal muscles of control and SMA mice (Fig. 3E–J). We showed that ex15-16 levels in SMA mutants as well as the effects of flunarizine on controls and mutants were muscle-specific. Indeed, a threefold reduction of ex15-16 levels was observed in SMA soleus while a ~5-fold increase was found in SMA plantaris when compared to controls (Fig. 3E–G). Flunarizine reduced the elevated ex15-16 levels in SMA plantaris but did not restore levels in SMA soleus. Likewise, to what was observed with the *Agrn* gene (Fig. 2H), SMA tibialis expressed as much *Cacna1h* mRNA levels as controls and increased similarly with the drug (Fig. 3G). Alternative ex25 levels were increased in SMA plantaris and tibialis compared to controls whereas they were reduced in SMA soleus. Flunarizine corrected the relative ex25 inclusion in mRNAs of SMA tibialis but increased it in control plantaris. The ratio ex2-3/ex15-16 remained low in flunarizine-treated SMA soleus and plantaris, indicating that the retention of the U12 intron persisted. Importantly, ex32-33/ex15-16 ratio showed a value of 1 in flunarizine-treated SMA plantaris, suggesting an increase of full-length mRNAs with the drug.

**CaV3.2 protein is enriched nearby NMJs and with flunarizine in SMA model mice.** *CaV3.2* current has been detected in murine neonatal myofibers. We then asked whether the distribution of *CaV3.2* in myofibers was impacted by flunarizine. To this end, we performed co-labelling immunofluorescence experiments using anti-type I MyHC7 (slow Type I fibers) and anti-CaV3.2 antibodies on serial sections of control and SMA plantaris at P5 and P10 (Fig. 4A, Supplemental Fig. 8). The SMA mice start developing symptoms at P543. The anti-CaV3.2 antibodies revealed a cytoplasmic and membranous labelling of myofibers. Reduction of total fluorescence in Type I myofibers between P5 and P10 was observed in all conditions, except for the vehicle-treated controls (Fig. 4B). The flunarizine-treated SMA type I fibers recovered cytoplasmic fluorescence at P10 compared to vehicle-treated SMA fibers. Flunarizine had no effects on total CaV3.2 fluorescence intensity of MyHC7-negative fibers (presumably rapid type II) in controls whereas an increase was observed in those SMA fibers. Thus, each fiber type responds differently to flunarizine.

Previous studies have shown CaV3.2 expression in motor neurons, blood vessels, muscle sensory afferents but not in Schwann cells, and all these cell types being present in muscle tissues. We focused here on the CaV3.2 membranous labelling of myofibers and NMJs looking at CaV3.2 labelled muscle sections at P10 counterstained with a fluorescent α-BTX to label AChRs (Fig. 5A–D). Our immunofluorescence experiments showed in P5 and P10 muscle sections that the immunoreactivity of CaV3.2 was close to that of the labelling with the fluorescent α-BTX, indicating that CaV3.2 is present nearby NMJs. We also showed that flunarizine enhanced CaV3.2 immunostaining nearby NMJs in SMA muscles (Fig. 5D). Thus, defective CaV3.2 distribution might contribute to SMA pathogenesis.

**Flunarizine modulates protein interactions in C2C12 myotubes.** To examine whether flunarizine modulates protein interaction between CaV3.2 and the agrin-LRP4-MuSK pathway, the C2C12 myotubes were treated with the drug, fixed and evaluated using in-cell co-immunoprecipitation protein ligation assay (PLA) with antibodies generated in different species and specific for each protein (Fig. 6). Focusing on proteins critical for the AChR clustering, we tested the following pairs: LRP4-MuSK, LRP4-CaV3.2, MuSK-integrin β1, MuSK-phosphotyrosines, integrins α11-β1 and Dyn2-integrin β1. The PLA positive signals with MuSK and phosphotyrosines, integrins α11-β1 and Dyn2-integrin β1. The PLA positive signals between two proteins in close proximity (≤40 nm) appeared as fluorescent dots that can be compared between DMSO- and flunarizine-treated C2C12 myotubes. Specific PLA signals were detected for all combinations. The positive signals with LRP4-CaV3.2 and LRP4-MuSK combinations were indicative of constitutive protein associations in C2C12 myotubes. We also found that flunarizine enhances the association of integrin-β1 with both MuSK and integrin-α11 whereas Dyn2-integrin-β1 association was reduced (Fig. 6B). Although MuSK phosphorylation was not detected in soluble immunoprecipitation protein fractions (Supplemental Fig. 10), PLA signals generated with MuSK and phosphotyrosines were markedly increased by flunarizine. Thus, flunarizine changes the close association(s) of key NMJ proteins.

**Flunarizine reduces Tnxis expression in skeletal muscles of SMA model mice.** Oxidative damages might also contribute to NMJ alterations in motoneuron diseases. Flunarizine reduces the pro-oxidant TXNIP in SMA patient fibroblasts like other calcium channel blockers do in pancreatic beta cells. Interestingly, *Tnxis* transcripts were upregulated in muscles with no satellite cells (SC) compared with SC replete muscles that is reminiscent of reduced SC number in muscles of SMA mice. We examined *Tnxis* expression in muscles of flunarizine-treated SMA mice (Fig. 7). Flunarizine reduced upregulated *Tnxis* mRNA levels in SMA muscles (Fig. 7A). Immunofluorescence co-labelling experiments with antibodies against TXNIP and MyHC7 (Type I fibers) revealed that all neonatal myofibers were TXNIP-positive with 2% being strongly labelled in controls while 16% were in the SMA soleus and reduced to 10% with flunarizine (Fig. 7B,C). This fits with high TXNIP expression in disused adult mouse and rat muscles. Thus, flunarizine reduces the elevated *Tnxis* mRNA levels in muscles of SMA mice.

**Discussion**

Three SMN-restoring therapies exist for SMA patients that improve survival and motor function, but yet patient response to treatments is variable. So, additional therapeutic targets should be identified to optimize existing SMA therapies or implement new ones. We have previously shown flunarizine to increase myofiber size, NMJ area and innervation in SMA model mice. We focused herein on skeletal muscles to assess flunarizine on NMJs.
We show that flunarizine can modulate agrin-signalling pathways, involved in NMJ formation and maturation, providing further insight into SMA pathogenesis.

Motoneuron disease mouse models and patients present NMJ defects\textsuperscript{75,76}. Synaptic dysfunction is an early event in SMA that generates changes in sensory-motor circuits\textsuperscript{77–79}. SMA is a retrograde neuropathology in which motoneuron degeneration begins at presynaptic NMJs and progresses to cell bodies\textsuperscript{15}. In severe SMA mouse models, motoneuron numbers are normal at birth and decrease around post-natal day 3 to 5\textsuperscript{80,81}. Post-natal NMJ

Figure 4. Flunarizine prevents the reduction of myofiber Cav3.2 immunolabelling during neonatal development in SMA mice. (A) Muscle fiber immunostaining with Cav3.2 (green) and MyHC7 (red) is shown for the plantaris from vehicle (V)- and flunarizine (FZ)-treated control and SMA mice at P5 and P10, respectively. The original images are presented in supplemental Fig. 8. (B) Normalized fluorescence intensity of individual muscle fibers for Cav3.2 detected in panel A is shown. Each symbol represents total value of fluorescence intensity in a fiber. The mean value of fluorescence is in supplemental Fig. 9. The distribution for the 4 groups of mice of MyHC7 immunolabelled or unlabeled fibers is compared using Student’s t-test. NS not significant (P > 0.05), *P < 0.05, **P < 0.01, ***P < 0.001 (3 mice per group). Scale bar, 50 μm.
maturation occurs during the first 3–4 weeks after birth. During this period, NMJs enlarge and their morphology changes from oval to perforated plaques to reach a pretzel-like shape26. Ca,3.2 current is high in perinatal myofibers, progressively decreases during the 3–4 weeks after birth and is undetectable in adult muscles, except during muscle regeneration and repair27. This developmental period has high demands for SMN and is when the motor phenotype caused by SMN deficiency can be rescued by SMN-based therapies83–85. It is also when SMN-independent therapies improve disease phenotype2,4,5,86–88.

Stimulated agrin/LRP4/MuSK pathway ameliorates SMA and amyotrophic lateral sclerosis (ALS)89. Agrin is a key molecule in post-synaptic NMJ biology. Changes in agrin isoforms impact on NMJ formation, maturation and stability90. Agrin gene has alternative spliced Y and Z exons. Isoforms with Z-sites (Z-) are synaptogenic at NMJs whereas isoforms without them (Z) have little AChR clustering activity in vivo90. Motoneurons express Z’- agrin while muscle cells do not. Z agrin has also functions: it can modulate AChR phosphorylation91, stimulate AChR gene expression92,93 and in certain conditions, induce AChR clustering60,94,95. Z’- agrin can bind myotube cell surface proteins, such as neural cell adhesion molecule, α-dystroglycan, laminins and integrins89,96–99. Z’- agrin can also bind these proteins, but the α-dystroglycan binds more strongly to Z’- agrin than Z’- agrin. Moreover, the Y-site changes the interactions, the Y’-Z’- agrin exhibiting the highest binding to α-dystroglycan90. The flunarizine-modulated Y-site also implicates α-dystroglycan activity to the response in SMA muscles.

At post-natal day 10, each skeletal muscle is very different in term of maturation. The embryonic MyHC persists for a time after birth. The soleus is the less mature muscle, as a larger proportion of embryonic fibers (60%) than in plantaris (30%) and tibialis (20%) is found3. Two Agrn promoters produce transcripts with either NtA or Tm first exon encoding secreted or transmembrane isoforms, respectively. Both motoneurons and myofibers express NtA exon, encoding a laminin-binding domain90. It has been shown that Smn-deficient motoneurons derived from SMA mice do not respond to laminin910, which is consistent with a reduction in NtA-agrin. The marked NtA reduction we observed also in SMA muscles indicates altered expression in both cell types. NtA and Tm exons are not equally affected in SMA muscles and respond differently to the drug in a development/muscle-specific manner, the more mature plantaris muscles behaving like controls. Tm exon in vivo is expressed almost exclusively by neurones27 and in muscles, by neurones innervating myofibers. Different Tm exon levels are observed among SMA muscles, suggesting a difference in skeletal motoneurons101 and/or in retrograde signals from the myofibers3.

Although a threshold Tm exon level correlates with flunarizine effects on NMJ size in SMA plantaris and tibialis83, additional mechanisms have to be considered to explain how it occurs in SMA plantaris (similar Tm levels between vehicle and flunarizine). It has been shown that the Y-site modulates Tm-agrin at excitatory synapse-like specializations in the central nervous system94. These authors proposed that an indirect interaction of Tm-agrin and LRP4 is required to cluster proteins at post-synaptic membrane. It is possible that Ca,3.2 could mediate indirect interactions. Other studies have shown that integrin-β1 could mediate the interaction between agrin and α-dystroglycan95,96. It is therefore tempting to speculate that Ca,3.2 might be an agrin co-receptor in early post-synaptic NMJ differentiation.

Regarding Cacna1h gene (encoding Ca,3.2), its U12-dependent intron splicing of Cacna1h gene is altered in SMA mice7. Motoneuron disease ALS and a severe congenital amyotrophy are associated with genetic Cacna1h variants as risk factors102–105. Interestingly, other types of calcium channels, namely P/Q-type, limit the localization and activation of MuSK and prevent AChR clustering in extra-synaptic domains of myofibers106. It is interesting that a blocker of T-type calcium channels can modulate the expression of Cacna1h at the RNA and protein levels. The reason might be the low selectivity of the drug for Ca,3.2 channels107. It is tempting to speculate that inhibition of T-type channels partially explains the flunarizine effects. The prolonged action of flunarizine on Cav3.2 current inhibition provides support for a role of this channel (Supplemental Fig. 7). Flunarizine might promote a feedback loop on Cacna1h expression by altering intracellular Ca,2+ levels that counteracts NMJ defects in SMA plantaris and tibialis. This is in agreement with the role of altered RNA metabolism in SMA and ALS108 and the effects of flunarizine on RNA splicing83. Our results have also broad relevance in other human diseases suggesting that flunarizine could be deleterious in young patients affected with a channelopathy caused by dominant-negative Ca,3.2 mutations109.

Calcium is a key regulator in skeletal muscle biology. AChR cluster formation and maintenance involved intracellular and extracellular calcium100,101. In murine muscles, Ca,3.2 current peaks around embryonic day E16 that coincide with the late embryonic NMJ maturation step. Indeed, aneural AChR clusters are present at E11-12.5, then innervation takes place (E12.5-E14) and aneural clusters disperse at E14-E17 and only innervated clusters remain on myofibers102. Myogenesis also occurs in this period. From E14.5 to birth, myoblasts adhere and fuse to primary fibers to form secondary myofibers103. Ca,3.2 current is activated in vitro when myoblasts fuse to form myotubes104. This may explain the reduced muscle size in SMA mice. Reduced number of myofibers in SMA soleus and plantaris105 correlates here with the reduced Cacna1h RNA levels. Flunarizine increases fiber size and number to reduce atrophy of SMA soleus, but only fiber size to correct atrophy in SMA plantaris106. Post-natal growth of muscle normally happens by increases in fiber area but not in fiber number107,114. These observations might point to a difference in myofiber types and their respective expression of Ca,3.2, as seen herein. The soleus presents a 50%–50% proportion of slow type I and fast type II fibers. The plantaris and tibialis contain a small proportion of type I fibers (10–20%) that disappears almost completely during postnatal development. Resting calcium levels are higher in slow than fast fibers. Therefore, calcium transients have different effects in either fibers due to the level differences in proteins controlling calcium homeostasis. Calcium can activate calmodulin-regulated phosphatase calcineurin and can activate calcium-dependent proteases. In slow myofibers, calcineurin regulates the nuclear translocation of transcription factor NFATc1 controlling the slow gene program. Furthermore, increased calcium influx through Ca,3.2 induces cardiac calcineurin/NFAT hypertrophic signalling115. Given that calcium handling is altered in SMA50, flunarizine might modulate the expression of other muscle genes still to be identified in the future.
is complex. RNA analysis identified Wnt4 and Wnt9a expression in C2C12 myotubes that are not changed by its potential localization nearby the NMJs during perinatal development. We can speculate but do not yet fully after birth, our findings point to the importance in investigating the drug effects during neonatal development in SMA mice. With a better understanding of the responses to flunarizine, it may be possible to extend...

Automated patch-clamp recordings were performed using the SyncroPatch 384PE from Nanion (München, Germany). Whole-cell experiments were performed at a holding potential of −100 mV at room temperature (18–22 °C). Currents were sampled at 20 kHz. For pharmacological assays, flunarizine solutions were prepared at various concentrations and applied to the bath of the recording chamber.

**Materials and methods**

**Cell culture, myotube treatment and immunodetection.** C2C12 muscle cells were grown on TPP culture dishes in DMEM-glutamax supplemented with 20% fetal calf serum (FCS) and penicillin and streptomycin (100 units/ml) and differentiated in DMEM-glutamax containing 2% horse serum. After 6 days in differentiation medium, myotubes were treated overnight with flunarizine (4 μM, as recommended by Sigma-Aldrich for Lopac library L6912, Cav3.2 calcium current IC₅₀) or DMSO (1 μl/ml) as schematically shown in Fig. 2a. For the experiments using blocking antibodies (1:100, according to the literature), they were added to the myotubes an hour before the molecules. For fluorescence microscopy, myotubes were rinsed with PBS and fixed for 15 min at room temperature with 3% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4 and rinsed in PBS. AChR clusters were visualized using Alexa Fluor 488 or 555-conjugated α-bungarotoxin (2 μg/ml, Molecular Probes). For western blotting, total protein extracts were resolved on 10% ProSieve 50 polyacrylamide gels (FMC Bioproducts, Rockland, ME) in Tris-Tricine running buffer, transferred to PVDF membranes (Millipore), and incubated with antibodies listed in Supplemental Table 1. After the washing step of the incubation with HRP-conjugated secondary antibodies, the proteins were visualized using chemiluminescence (Amersham ECL, GE Healthcare) and quantify using ImageJ's gel analysis. For electrophysiological recordings, HEK293 stably expressing hCaV3.2 were cultivated high glucose DMEM supplemented with 10% FBS, 1 mM L-glutamine, 1 mM penicillin/streptomycin and completed with 800 μg/ml geneticin G418.

**Electrophysiological recordings of Ca₃.2.** For electrophysiological recordings, trypsinized cells were centrifuged at 800 rpm for 4 min and resuspended (= 350,000 cells ml⁻¹) in patch-clamp extracellular solution. Whole-cell recordings were used to investigate the effects of flunarizine on Cav3.2 expressing HEK293 cells. Automated patch-clamp recordings were performed using the SyncroPatch 384PE from Nanion (München, Germany). Chips with medium resistance single holes (n = 384) were used for electrophysiological recordings. Pulse generation and data collection were performed with the PatchControl384 v1.9.7 software (Nanion) and the Biomek interface (Beckman Coulter). Whole-cell recordings were conducted according to the recommended procedures of Nanion. Cells were stored in a cell hotel reservoir at 10 °C with shaking speed at 60 RPM. After initiating the experiment, cell catch, sealing, whole-cell formation, liquid application, recording, and data acquisition were all performed sequentially and automatically. The intracellular solution contained (in mM): 60 NMDG, 80 NaCl, 4 KCl, 1 NaATP, 1 MgCl₂, 5 Glucose, and 10 HEPES (pH 7.4 with NaOH). Whole-cell experiments were performed at a holding potential of −100 mV at room temperature (18–22 °C). Currents were sampled at 20 kHz. For pharmacological assays, flunarizine solutions were prepared at various...
concentrations in the extracellular solution supplemented with 0.3% bovine serum albumin, and distributed in 384-well compound plates according to a pre-established plate plan. This distribution within compound plates could not be randomized since it subsequently would excessively complexify the methods of analyses. The working compound solution was diluted 3 times in the patch-clamp recording well by adding 30 to 60 µL external solution to reach the final reported concentration and the test volume of 90 µL. For the control groups, vehicle solutions were extracellular containing 0.3% BSA and 0.1% DMSO (which was similar to the DMSO concentration in the 10 µM flunarizine solution). Flunarizine effects were measured all along during a 10-min application
Figure 6. Visualization of key NMJ protein interactions in flunarizine-treated C2C12 myotubes. (A) In situ PLA detection of endogenous protein interactions in C2C12 myotubes with specific antibodies for the following protein pairs: LRP4-MuSK, LRP4-Cav3.2, MuSK-integrin β1, integrin α11-β1, Dyn2-integrin β1 and MuSK-phosphotyrosines. As negative controls, one primary antibody is omitted or replaced by a negative control antibody (DAKO). The PLA positive signals are shown as bright dots. (B) Fold change in mean fluorescence intensity of individual C2C12 myotubes detected in panel A is shown. The number of myotubes analysed with ImageJ is found in supplemental Table 3. Scale bar, 30 μm.
Figure 7. Flunarizine reduces the increased Tnixp expression levels in skeletal muscles of SMA model mice. (A) Tnixp RNA levels in the soleus, plantaris and tibialis in vehicle- (V) and flunarizine (FZ)-treated controls (Ctrl) and SMA model mice. Vehicle-treated control (Ctrl V) is given an arbitrary value of 1. NS not significant (P > 0.05), *P < 0.05, **P < 0.01, ***P < 0.001, Student's t test (3 mice per group). (B) Muscle fiber immunostaining with anti-TXNIP (green) and anti-MyHC7 (red) antibodies is shown for the soleus from vehicle- and flunarizine-treated control and SMA mice at P11. (C) Columns represent the percentage of fibers strongly TXNIP-positive (3 mice per group). Error bars represent SD from the mean values. Scale bar, 30 μm.
time with a single step protocol containing a 50 ms – 100 mV holding potential period followed by a 400 ms pulse at –10 mV. This single step protocol was repeated with a 10 s interval.

**Animal procedure and muscle tissue experiments.** Animal experiments were performed in accordance with the ARRIVE guidelines and the protocols approved by the Université de Paris animal protection committee (CEEA 34) respecting the European and French guidelines for care and uses of laboratory animals (2010/63/EU) under the reference numbers 01246.02 and B75-06-07. The Taiwanese SMA mice were injected daily from birth with either flunarizine (500 µg/ml, 1 µl/g) or vehicle (1% DMSO in saline solution) as described. The SMA mice are on FVB/N background (Janvier, Le Genest-St-Ise, France) and have been backcrossed for 10 generations as described. A number was assigned to each animal, and unless stated otherwise, the experiments were performed blinded for genotype, treatment and molecular and cellular studies. The group allocation was disclosed for the analyses. No exclusion criteria were used. Both females and males were included in the study. The severe Taiwanese SMA model (Smnko/ko, SMN2tg/0) and the corresponding control heterozygous (Smnko/wt, SMN2tg/+) mice were produced in the same litter. Based on our previous results, we studied three mice per experimental group to minimize the number of animals used. The four groups were vehicle- and flunarizine-treated control and SMA control mice for a total of 59 mice. The mice were genotyped with PCR primers S1, S2, H1, 2B and 2F. Briefly, a multiplex PCR reaction was set up with 1 µl DNA (20 ng), 5 µl 5X green GoTaq flexi buffer, 2.5 µl 25 mM MgCl2 (2.5 mM final), 1 µl 10 mM dNTPs mixture (2.5 mM each dNTP, 200 µM final), 1.5 µl 10 µM S2 forward primer (0.6 µM final), 0.75 µl 10 µM 2F forward primer (0.3 µM final), 0.75 µl 10 µM S1, H1, 2B reverse primers (0.3 µM final) and 0.15 µl GoTaq DNA polymerase (Promega). Amplification conditions were as following: 5 min at 95 °C then 30 s at 95 °C, 1 min at 58 °C, 45 s at 72 °C for 33 cycles and a final extension step of 5 min at 72 °C. The PCR products S2-S1 (1150 bp), S2-H1 (950 bp) and 2F-2B (478 bp) were analysed on a 1% agarose gel. SMA mutants and their heterozygote littermates were anesthetized by intraperitoneal injections of pentobarbital (64 mg/kg) and decapitated at P5 or P10. Three skeletal muscles (soleus, plantaris, tibialis) were dissected, snap-frozen and stored at −80 °C. The immunofluorescence experiments on muscle cryosections (10 µm) were performed as we previously described. Briefly, the sections were fixed in 4% PFA in PBS for 20 min, blocked in 20% FCS or 4% BSA for 30 min and incubated with primary antibodies overnight at 4 °C. After 1 h in PBS-Tween (0.1%), the sections were incubated for 2 h with secondary antibodies. After bis-benzimide (or DAPI) staining and washes, the sections were mounted with fluoromount-G solution. The antibodies used are listed in Supplemental Table 1.

**Immunofluorescence microscopy image acquisition, processing and analysis.** Images were taken using an ORCA Flash 2.8 camera (Hamamatsu Photonic) mounted on an epifluorescence microscope system (AxioObserver Z1, ZEISS). ZEN and ImageJ softwares were used to acquire and analyse the images, respectively. Briefly, regions of interest (ROIs) were randomly selected and manually drawn using the brightfield channel images with ImageJ. For the fluorescence intensity analyses of muscle fibers, first the threshold level was adjusted to remove any background signal and the mean gray values of Cav3.2 labelled muscle fibers were measured for fibers of either type I-labelled or –unlabelled fibers (presumably type II) for each group of mice. The size of AChR clusters in C2C12 myotubes was determined with ImageJ by the measure of AChR aggregates (≥ 6 to 10 µm) in randomly selected 20× microscope fields (40 fields per experiment, n ≥ 3 independent experiments) or from a hundred fibers per experiment (n = 3).

**In situ proximity ligation assay.** In situ proximity ligation assay (PLA) was performed as we described. Briefly, C2C12 cells were fixed and permeabilized as above. Primary antibodies (Supplemental Table 1) were diluted in the antibody diluent (Duolink II kit, Sigma-Aldrich) and incubated overnight at 4 °C. Negative controls consisted of using a single primary antibody. The cells were washed twice for 5 min in Tris-buffered saline with 0.05% Tween. The PLA probes (mouse-PLUS and rabbit-MINUS) were incubated for 1-h at 37 °C. Subsequent steps were performed using the detection reagent Orange. Signals were viewed as dots with a fluorescence microscope and analysed with ImageJ.

**RNA preparation and expression analyses.** Total RNA was extracted from tissues and cell cultures using Trizol Reagent (Ambion) and treated with a RQ1 RNase-free DNase (Promega). One µg of RNA was used to generate cDNA with miScript II RT kit (Qiagen) for snRNA analyses and Superscript III (Invitrogen) for the other genes. Quantitative real-time PCR was performed in triplicate using the primers listed in Supplemental Table 2 with SYBR Green ROX mix (Thermo Scientific) on either Applied Biosystems 7500 fast system or Bio-Rad CFX384. The normalized expression levels were calculated according to the ΔΔCt method. The snRNA, 5 S, 5.8 S, Rpl13a, Ppia, Gapdh, Myh4 and Z’ Agrn primers and their analyses have been previously described. Additional primers have been designed using the free primer design tools from eurofins (eurofinsgenomics.eu) and validated according to the MIQE guidelines.

**Statistical analyses.** At least three independent experiments were presented as the mean ± SD or SEM. Statistical analyses were conducted using the non-parametric Kruskal Wallis followed by Dunn’s multiple comparison rank test or two-way ANOVA followed by Tukey’s multiple comparisons test or Student t-test (GraphPad). The test used was indicated in the figure legends. The significant threshold was 5%.

**Data availability** The detailed calculations made during and/or analysed during the current study are available from the corresponding author on reasonable request.
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**Author contributions**

P.D., D.S., B.S., S.D.W., M.D.W. and S.L. performed experiments. P.D., D.S., B.S., S.D.W., M.D.W. and S.L. conducted analyses. S.L. conceived the project and wrote the manuscript. All authors commented on the manuscript.

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**Competing interests**

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