

Crosstalk between Notch and Sonic hedgehog signaling in a mouse model of amyotrophic lateral sclerosis

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The developmental morphogen Sonic hedgehog (Shh) may continue to play a sustaining role in adult motor neurons, of potential relevance to motor neuron diseases including amyotrophic lateral sclerosis. The Shh signaling pathway is incompletely understood and interactions with other signaling pathways are possible. We focus here on Notch, and first show that there is an almost linear reduction in light output from a Gli reporter in Shh Light II cells in the presence of increasing concentrations of the Notch inhibitor DAPT ($r^2 = 0.982$). Second, in the spinal cord of mutant superoxide dismutase mice, but not control mice, a key marker of Notch signaling changes with age. Before the onset of clinical signs, the Notch intracellular domain is expressed predominantly in motor neurons, but by 125 days of age, Notch intracellular domain expression is markedly reduced in motor neurons and increased in neighboring astroglia. Third, there is a parallel reduction in Gli protein expression in mutant superoxide dismutase mouse spinal motor neurons, consistent with the observed reduction in

Notch signaling and also a redistribution of Gli away from the nucleus. Thus, there is a reduction in motor neuronal Notch signaling and associated changes in Shh signaling, occurring coincidentally with disease expression, that may contribute toward the dysfunction and death of motor neurons in amyotrophic lateral sclerosis. *NeuroReport* 28:141–148 Copyright © 2017 The Author(s). Published by Wolters Kluwer Health, Inc.

NeuroReport 2017, 28:141–148

Keywords: amyotrophic lateral sclerosis, DAPT, G93A SOD1 mice, Light II cells, Notch, Sonic hedgehog

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Received 19 October 2016 accepted 28 November 2016

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by a progressive and ultimately fatal weakness of voluntary muscle. Most patients have sporadic disease, although about 5–10% of patients have familial amyotrophic lateral sclerosis (FALS). To date, mutations in over 30 different genes have been implicated in FALS [1], nearly all autosomal dominant in expression. The clinical presentation and neuropathology are mostly the same, and we do not understand how sporadic ALS and multiple different genetic forms of FALS lead to the same clinical phenotype. In the case of FALS, it is also not understood how a disease characterized by a dominant genetic mutation in all cells can be asymptomatic until mid-adult life, and then lead to rapidly progressive dysfunction of motor neurons and death sometimes within months.

The pathophysiological processes leading to motor neuronal dysfunction and death are poorly understood, even for FALS, where we know the underlying genetic abnormality, and may include oxidative and excitotoxic stress to motor neurons in the anterior horn of the spinal

cord and cortical motor strip, mitochondrial dysfunction, glial activation, and terminal apoptosis. Unfortunately, treatments targeting these processes have failed to show benefit in human studies. There is disrupted RNA processing and pathological protein misfolding, and almost certainly other key elements remain unknown [2].

We have previously explored the possibility that small molecules necessary for the differentiation of embryonic motor neurons, particularly Sonic hedgehog (Shh), might continue to play a supportive role to motor neurons in adult life and might be implicated in the pathogenesis of ALS. Differentiated cell cultures with motor neuronal properties (N2A, NSC34, and Sy5y) stably transfected with a human mutant superoxide dismutase (mSOD) are unduly susceptible to hypoxic and excitotoxic stress, and cell death can be mitigated or potentiated, respectively, by the addition of Shh or Shh antagonists to the culture medium [3]. The primary cilium, an organelle necessary for Shh signaling [4], progressively disappears from motor neurons in the anterior horn of adult transgenic G93A SOD1 (mSOD) mice, but not from other spinal neurons or from control mice [5]. Shh has been implicated in the pathogenesis and suggested as treatment of other neurodegenerative diseases including Parkinson's and Alzheimer's disease, in part but not exclusively because of its role in the maintenance of stem cell niches in adult organisms [6]. These observations all suggest that the

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Shh pathway retains physiological relevance in normal adult motor neurons and may be defective in neurodegenerative disease including ALS.

Canonical Shh signaling [7] occurs following binding of Shh to its transmembrane receptor patched and three coreceptors (GAS1, CDON, and BOC), an action that releases smoothed to translocate to the primary cilium, where it is phosphorylated and subsequently affects the metabolism of the three downstream Gli effector proteins (Gli1, 2, and 3). In the absence of Shh, the Gli proteins are sequestered by the suppressors KiF7 and SUFU, phosphorylated by GSK3 β , PKA, and CK1, and cleaved into translational repressors (e.g. Gli3-R) or degraded in the proteasome. In the presence of Shh, Gli2 and Gli3 in particular remain uncleaved and displace the repressor forms from nuclear Gli promoter sites to increase transcription of Shh target genes. However, the exact mechanism of Shh signaling is incompletely understood and other signaling pathways may modulate Shh signaling. A standard measurement of Shh bioactivity is the measurement of firefly luciferase activity driven by a Gli promoter in modified mouse fibroblasts (Shh Light II cells). Interestingly, Shh Light II cells need to be grown to near confluence before Shh activity is observed, which suggested to us that Notch, a cell contact signal, might modulate Shh activity.

Notch signaling is fundamental in controlling cell fate decisions, migration, growth, synaptic plasticity, and neuronal survival [8]. Abnormal activation of Notch signaling has been indicated in several neurodegenerative diseases, including ALS [9], Alzheimer's disease [10], and Pick's disease [11]. Notch signaling occurs in a cell contact-dependent manner and is initiated when the Notch receptor (Notch 1–4) binds to the transmembrane ligand, jagged/delta (delta-like 1, 3, 4; or jagged 1 or 2), on the surface of an adjacent cell [12]. This results in the sequential cleavage by TACE (tumor necrosis factor- α ADAM metalloprotease converting enzyme) of the extracellular Notch domain and cleavage by γ secretase of the intramembranous domain, and then diffusion of the Notch intracellular domain (NICD) to the nucleus, where it binds to CSL [CBF1/Su(H)/Lag-1] promoter regions to regulate transcription of Notch-target genes (HES family, cyclin D3, p21, myc, etc.). There are several additional signaling modifiers (including deltex, numb, mastermind-like 1, recombination signal bonding protein for immunoglobulin κ J, E3 ubiquitin protein ligase, F box only protein 7). NICD is readily observed by immunohistochemistry and is commonly used as an index of Notch activity in the cell.

In this paper, we show that Notch directly modulates Shh signaling, as assessed by measurement of Shh bioactivity in Shh Light II cells in the presence of increasing concentrations of the Notch inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine-t-butyl ester (DAPT). We show

that Notch signaling as measured by NICD is decreased in spinal cord motor neurons of mSOD mice coincident with disease expression. Finally, we show that Gli2 and Gli3 immunohistochemistry is altered in mSOD spinal motor neurons, consistent with a downregulation of Shh signaling because of changes in Notch.

Materials and methods

Shh Light II cell culture with the Notch inhibitor and the Dual-Glo luciferase assay

Shh Light II cells were obtained from the Johns Hopkins University cell center. These cells were initially developed as Shh reporter cells by stably transfecting NIH/3T3 mouse fibroblasts with a Gli-responsive firefly luciferase reporter, and a constitutively expressed renilla luciferase under control of the thymidine kinase promoter, along with antibiotic selection markers [4].) Shh Light II cells were grown to full confluence in 96-well luminescence plates (Cat #655073; Greiner BioOne CellStar, Monroe, North Carolina, USA) in Dulbecco's modified Eagle's medium with 0.5% fetal calf serum, 5 mM HEPES buffer, and Zeocin, (Invitrogen, Burlington, Ontario, Canada) and then cultured for 24 h in the presence of 0, 2.5, 5, or 10 μ M DAPT (Sigma Aldrich, Sigma-Aldrich Canada Co. Oakville, Ontario, Canada), a γ secretase inhibitor that blocks intracellular Notch signaling. The cells were then cultured for a further 24 h in the continued presence of DAPT, and a titer range of Shh (Sigma Aldrich; 0, 0.25, 0.50, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 2.75 μ g/ml culture medium). Luminescence was then measured in a Tecan M-1000 Pro luminometer (Tecan, Männedorf, Switzerland) for firefly and renilla luciferase following the Promega Dual-Glo luciferase assay protocol (Promega, Madison, Wisconsin, USA).

Animals and ethics statement

Male transgenic mSOD mice [B6SJL-TgN(SOD1-G93A)1Gur] were purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and harem bred with female wild-type (WT) B6SJL mice to establish a colony. Offspring were genotyped for the mSOD transgene using PCR of DNA extracted from tail samples using the protocol outlined on the Jackson website. Animals were housed 1–5 per cage with a 12-h light/dark cycle. All mice were fed standard murine chow and water ad libitum. Sixty days, 117 days, and endpoint of mice were used. The experimental protocol was approved by the McMaster University Animal Research Ethics Board and was carried out in accordance with guidelines of the Canadian Council on Animal Care.

Tissue preparation

For immunofluorescent staining, mice were anesthetized with isoflurane inhalation and perfused transcardially with 50 ml of PBS, followed by 50 ml of 4% paraformaldehyde. The spinal column was removed and postfixed with 4%

paraformaldehyde at 4°C overnight, and then transferred to a 30% sucrose solution until saturated. Next, lumbar spinal cords were removed carefully using a dissection microscope, frozen, embedded in an optimal cutting temperature solution, and stored at -80°C until sectioning. Transverse sections at 40 µm (free floating sections) or at 14 µm (thin sections) were cut using a cryostat. All free floating sections were collected in series of six sequential sections and kept at -20°C in a cryoprotectant containing 25% glycerin, 25% ethylene glycol, and 0.05 M phosphate buffer. Thin sections were kept at -80°C.

Immunofluorescent staining

The entire lumbar L3 segment from a one-in-six series of free floating sections was examined. This resulted in about 5–7 sections examined for the L3 region for each animal. Immunofluorescent staining was performed exclusively on free floating sections as described previously [5], except for the animals at 125 days (thin sections of 14 µm were used to determine the cell types of NICD-positive cells). Briefly, after rinsing in PBS and blocking with 5% normal goat serum (Vector Laboratories, Burlington, Ontario, Canada), sections were incubated with the primary antibody overnight at 4°C. The primary antibodies used include mouse monoclonal anti-NeuN (1:500, MAB377; Chemicon, Temecula, California, USA), rabbit polyclonal anti-NICD (1:200, 07-1232; Millipore, Temecula, California, USA), rat anti-CD11b (1:100, MAB1387Z; Millipore, Temecula, California, USA), chicken anti-GFAP (1:200, AB5541; Millipore, Temecula, California, USA), rabbit polyclonal anti-Gli2 (1:200, GTX27195; GeneTex International, Irvine, California, USA), and rabbit polyclonal anti-Gli3 (1:200, ab6050; Abcam, Cambridge, Massachusetts, USA). The next day, sections were rinsed in PBS and incubated with secondary antibodies for 4 h at 4°C. All secondary antibodies were purchased from Molecular Probes (1:500; Molecular Probes, Carlsbad, California, USA). These included Alexa Fluor 488 goat anti-mouse highly cross-adsorbed (A11029) or anti-rat antibody, Alexa Fluor 568 goat anti-rabbit highly cross-adsorbed antibody (A11036), and Alexa Fluor 647 goat anti-chicken. Sections were then rinsed with PBS for several times and mounted on slides and coverslipped with ProLong gold antifade reagent with 4',6-diamidino-2-phenylindole (Molecular Probes, Carlsbad, California, USA).

Immunofluorescent microscopy analysis

The L3 spinal cord (5–7 sections per animal, 3–4 animals per age/group) was analyzed by widefield deconvolution microscopy (Leica DMI 6000B; Leica, Wetzlar, Germany). (The L3 level was chosen as mSOD animals develop a hind limb paralysis, with the brunt of the neuropathology observed at this level). For each section, images were captured with a digital camera using a ×20 objective lens (Hamamatsu Orca ER-AG, Bridgewater, NJ) and Velocity 4 Acquisition Software (PerkinElmer, Waltham, Massachusetts). The combination of the

neuronal marker NeuN and a cell size more than 20 µm was used to identify motor neurons in the ventral horn of the spinal cord. We analyzed all motor neurons on both sides of the cord. All motor neurons of WT mice showed intense cytoplasmic NICD staining at 60 days. We quantified the percentage of motor neurons showing a similar NICD signal intensity for WT mice at 117 and 138 days, and for mSOD mice at 60, 117, and 138 days, counting all motor neurons on both sides of the L3 cord ($n=2$ for each group).

Statistical analysis

For the Dual-Glo luciferase assay, the results are reported as the ratio of firefly luciferase activity to renilla luciferase activity, which serves as an internal control. Each experimental point was conducted in triplicate and graphed as the mean ± SE. The values for each of the four experimental DAPT conditions were curve fitted with a sigmoidal function and the plateaus were compared using linear regression (GraphPad Prism4 software). A nonzero slope of the regression line was tested using the *F* statistic (GraphPad Prism4 software).

Results

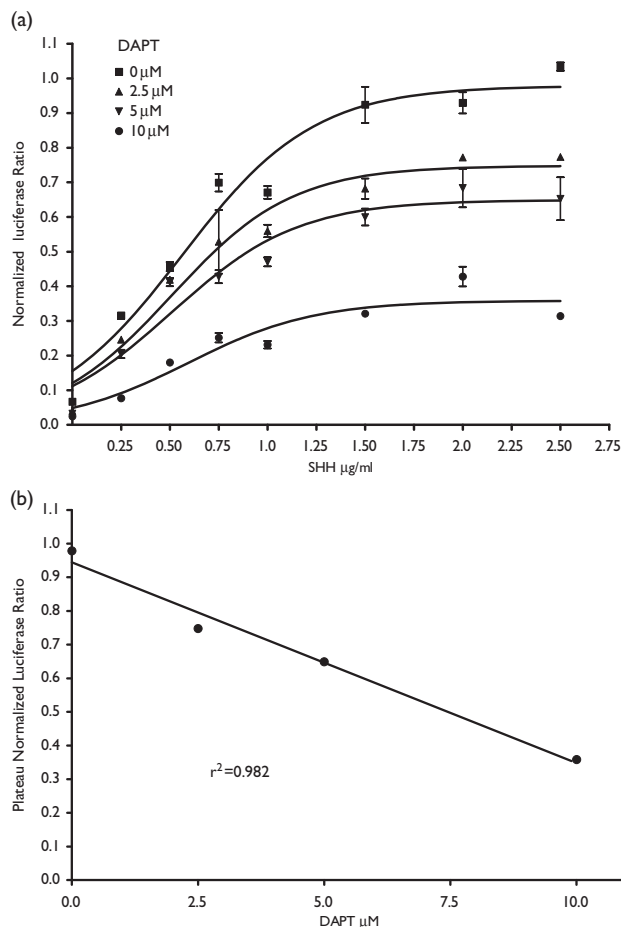
Notch inhibition and Shh signaling

Without the addition of DAPT, Shh Light II cells show an ~10-fold increase in luminescence with increasing concentrations of Shh, eventually plateauing at and above 1.50 µg/ml Shh at a value of 0.98 ± 0.03 . Increasing doses for DAPT progressively ablated the Shh effect on the Light II cells (Fig. 1a), such that the plateau was 0.75 ± 0.03 at 2.5 µM DAPT, 0.65 ± 0.03 at 5 µM DAPT, and 0.36 ± 0.02 at 10 µM DAPT. The decrease in Shh plateau with increasing DAPT is approximately linear over these DAPT concentrations ($r^2=0.98$; $F=109.7$, $P=0.009$) (Fig. 1b).

Notch signaling in the spinal cord

At 60 days, NICD was observed in L3 spinal motor neurons of both WT (Fig. 2a) and mSOD mice (Fig. 2b), with similar staining intensity. At 117 days, only faint staining of NICD was observed in motor neurons of mSOD mice (Fig. 2d) compared with more robust staining in motor neurons of WT mice (Fig. 2c). Very faint staining was observed in mSOD astroglia and no staining was observed in WT astroglia or microglia (data not shown). However, at 125 days, there was a major change. Although WT animals still showed predominantly motor neuronal staining (Fig. 2e), mSOD animals showed a marked reduction in motor neuron numbers and motor neuronal NICD staining, but a large increase in astroglial NICD (GFAP+ cells stained with NICD). This is shown in Fig. 2f, where the merged channel shows a purple color indicating an overlap of NICD and GFAP in mSOD mice. Microglia (CD11b+ cells) were increased in mSOD animals, but there was no microglial staining with NICD. The

Fig. 1



Notch inhibition of Shh Light II cells. Without the addition of DAPT, Shh Light II cells show increased luminescence with increasing concentrations of Shh, eventually plateauing at and above 1.50 $\mu\text{g/ml}$ Shh at a value of 0.98 ± 0.03 . Increasing doses of DAPT (2.5, 5, 10 μM) progressively ablated the effect of Shh on Light II cells, such that the plateau luminescence ratio (firefly/renilla) was 0.75 ± 0.03 at 2.5 μM DAPT, 0.65 ± 0.03 at 5 μM DAPT, and 0.36 ± 0.02 at 10 μM DAPT (a). The decrease in the Shh plateau with increasing DAPT is approximately linear over these DAPT concentrations ($r^2 = 0.98$; $F = 109.7$, $P = 0.009$) (b). DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine-t-butyl ester; Shh, Sonic hedgehog.

reduction of NICD staining in mSOD motor neurons was confirmed in endpoint mice at 138 days (Fig. 2h). Mice at 60 days showed intense cytoplasmic NICD staining of all motor neurons in the L3 spinal cord, and at 117 and 138 days, the percentage of motor neurons showing similar intense staining was mostly preserved at 93 ± 1.7 and $92 \pm 1.7\%$, respectively. However, there was a considerable reduction in the percentage of mSOD spinal motor neurons showing NICD staining: from $70 \pm 3.4\%$ at 60 days to 48 ± 2.5 and $24 \pm 2.0\%$ at 117 and 138 days, respectively.

Gli signaling in the spinal cord

The intensity of Gli2 staining was reduced in spinal motor neurons in mSOD mice compared with age-

matched WT mice, an effect that increased with age. For all age groups of WT mice, motor neuronal Gli2 staining was predominantly nuclear (Fig. 3a,c,e). However, in mSOD mice, Gli2 staining was redistributed away from the nucleus to the cytoplasm of motor neurons, especially with age (Fig. 3b,d,f).

A similar picture emerged for Gli3. Gli3 staining in WT mice was predominantly nuclear in spinal motor neurons at all ages (Fig. 4a,c,e). mSOD mice in the 60 days group (Fig. 4b) showed a distribution and intensity of Gli3 similar to that of age-matched WT controls, but there was a cytoplasmic redistribution at other ages and the intensity of Gli3 staining was markedly reduced at 117 days and had almost disappeared in the 138 days group (Fig. 4d,f).

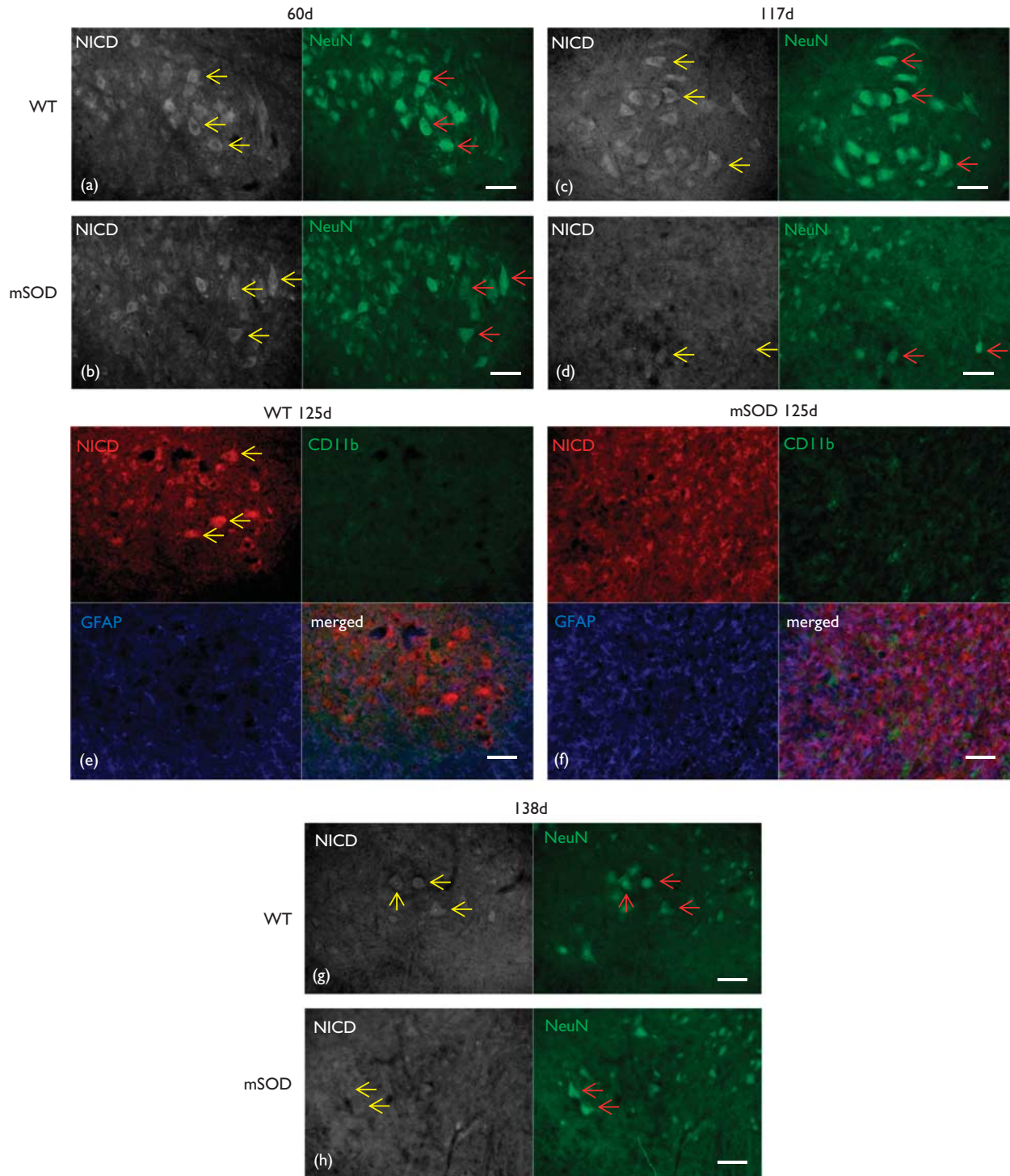
Discussion

DAPT, an effective and widely used inhibitor of Notch signaling [13], inhibits Shh signaling in Shh Light II cells in a dose-dependent and almost linear manner. The magnitude of the effect is important and at 10 μM DAPT, the effect of Shh on the Gli reporter is reduced by more than 50%. Shh Light II cells constitutively express renilla luciferase, which serves as an internal control for any generalized effect on cell viability or function because of DAPT. DAPT is a gamma secretase inhibitor, and as gamma secretase is implicated in processing other proteins besides Notch (e.g. APP, LDL receptor-related protein, E cadherin, ErbB4), additional effects on Shh signaling beyond Notch inhibition are possible. However, Notch signaling depends on cell-to-cell contact and Light II cells must be grown to confluence before effective Shh signaling occurs [14], strongly suggesting that Notch inhibition is the primary explanation for the reduction in Shh signaling observed with DAPT. Consistent with this, recent studies have shown that Notch signaling enables the translocation of smoothed to the primary cilium [15,16], an event essential to normal Shh signaling [4].

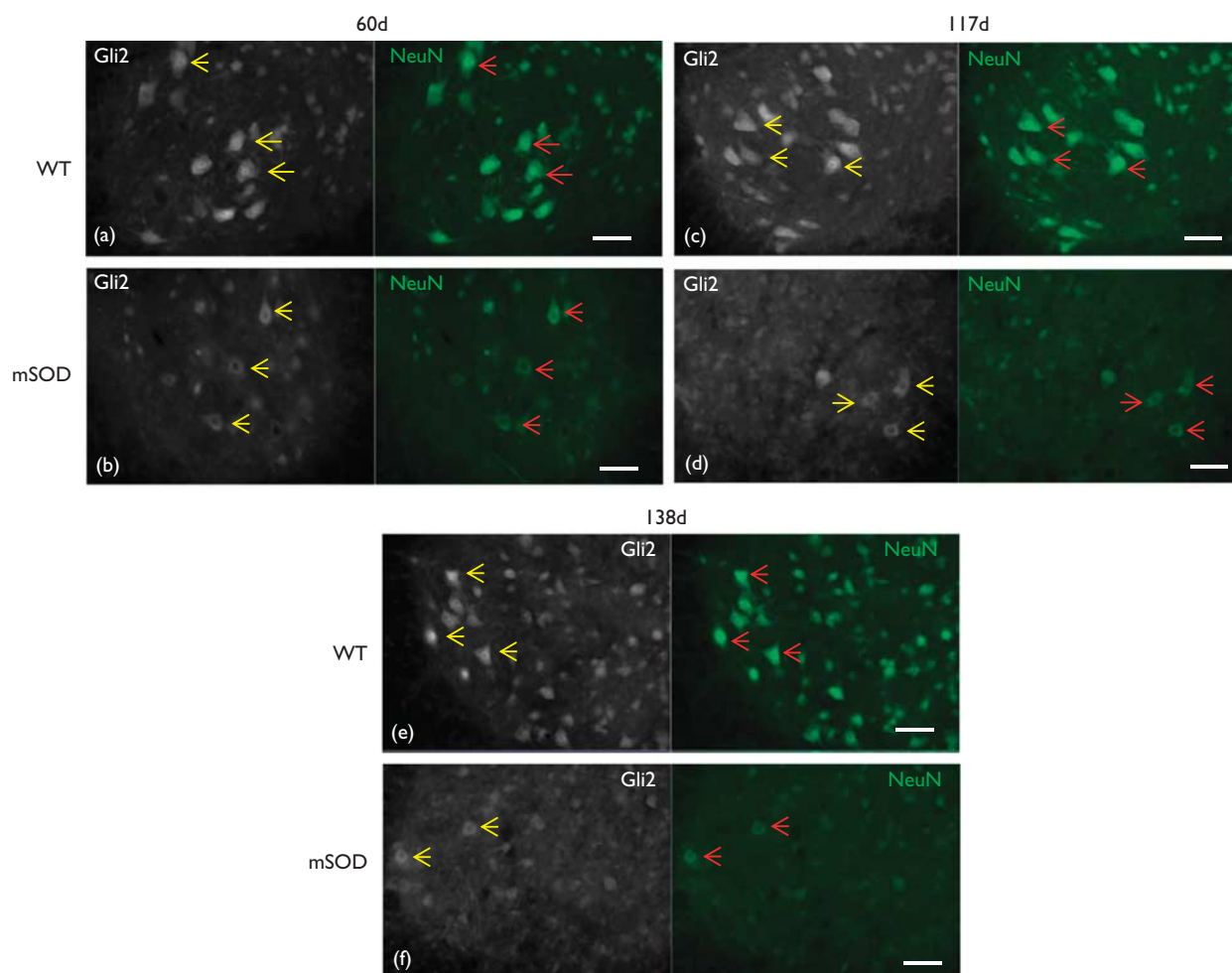
We observed important changes in Notch signaling as measured by NICD in the spinal cord of mSOD mice, but not WT mice. Moreover, these changes coincide with disease expression in the mice, which occurs roughly between 90 and 120 days of age. Over this age span, NICD is progressively reduced in motor neurons and increased in astroglia. We sought to understand whether the reduction in Notch signaling in motor neurons was reflected in changes in Shh signaling as might be expected.

Indeed, we observed a reduction in Gli2 and Gli3 protein levels in the motor neurons in parallel with the decrease in NICD. Although consistent with our expectation, other factors may contribute toward changes in Shh signaling besides impaired Notch signaling. As a case in point, we observed a redistribution of Gli immunocytochemistry

Fig. 2



Representative images in immunofluorescent NICD staining in motor neurons of lumbar spinal cords in WT and mSOD mice. At 60 days, NICD (yellow arrows) was observed in L3 spinal motor neurons (red arrows) of both WT (a) and mSOD mice (b), with similar staining intensity. At 117 days, faint staining of NICD (yellow arrows) was observed in motor neurons of mSOD (d), whereas staining in motor neurons of WT mice was unchanged (c). At 125 days, WT animals still showed predominantly motor neuronal staining [red channel (e)], but mSOD animals showed a large increase in astroglial NICD, but not microglia NICD [overlap of red and blue channels (f)]. Motor neuronal NICD staining was almost absent. The reduction on NICD staining in motor neurons was confirmed in endpoint mSOD mice at 138 days (h) compared with WT controls (g). Scale bar = 50 μm. mSOD, mutant superoxide dismutase; NICD, Notch intracellular domain; WT, wild type.

Fig. 3

Representative images of immunofluorescent Gli2 staining in motor neurons of the lumbar spinal cord in WT and mSOD mice. For all age groups of WT mice, Gli2 staining (yellow arrows) was observed predominantly in the nucleus and less in the cytoplasm (a, c, and e). However, Gli2 staining (yellow arrows) was redistributed from the nucleus to the cytoplasm in mSOD mice (b, d, and f). The intensity of Gli2 staining was also reduced compared with age-matched WT controls. Scale bar = 50 μm. mSOD, mutant superoxide dismutase; WT, wild type.

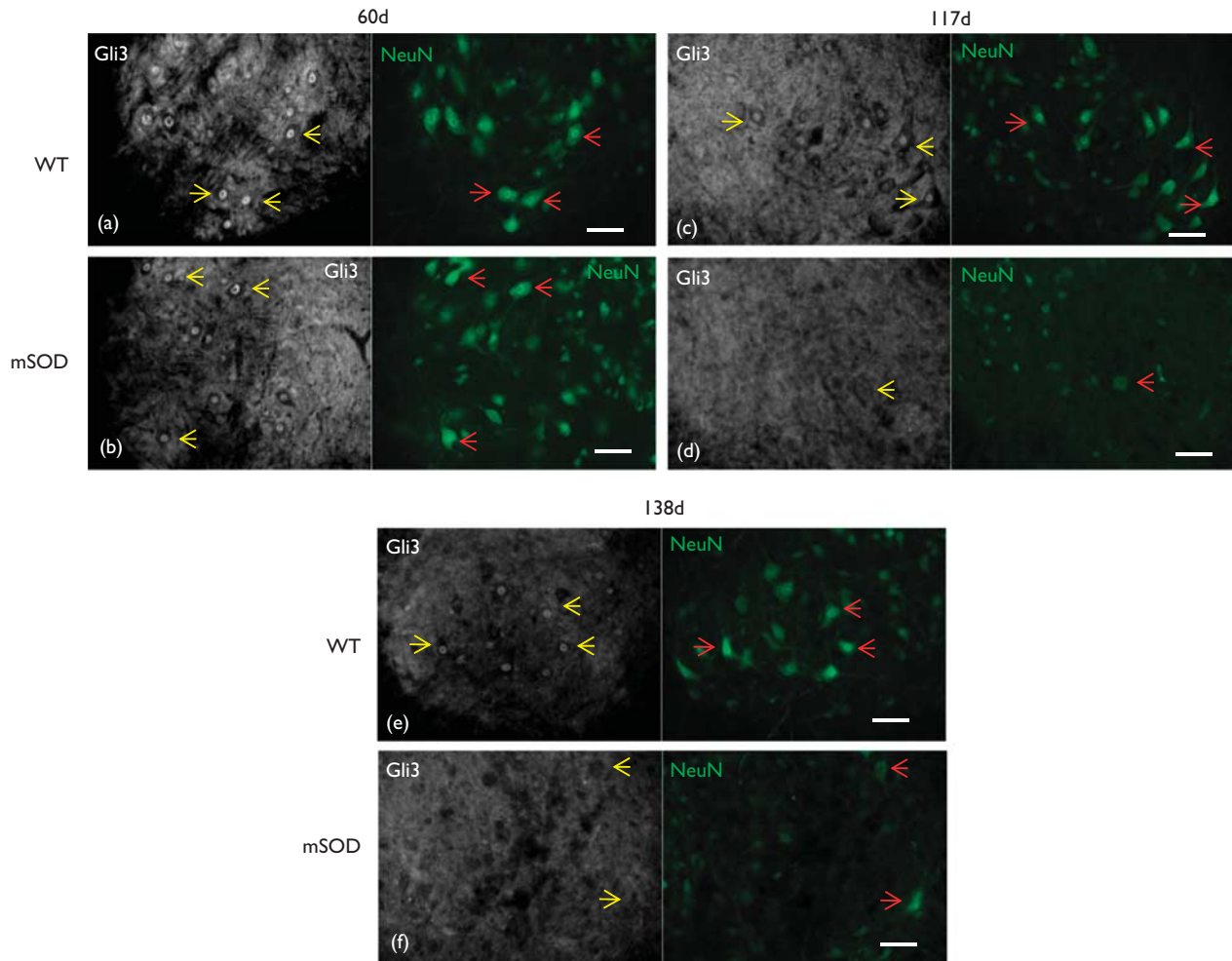
away from the nucleus and to the cytoplasm, and as the Gli proteins act as transcriptional regulators by binding to nuclear promoter elements, this relocation could be expected to further reduce Gli activity. However, this redistribution is more likely reflective of a general nucleocytoplasmic transport abnormality in ALS than an effect of Notch depletion. Some idea of the relative importance of Notch depletion alone could arise from measurements of Shh signaling in motor neurons of mSOD mice after conditional knockdown of Notch, but these experiments would be difficult to conduct and interpret because of the essential and widespread nature of Notch signaling.

The prominent NICD staining in motor neurons of mSOD mice before clinical disease was not fully anticipated as during neurogenesis, Notch ligand is expressed

in motor neuron progenitors and Notch receptor in supporting glia in most, but not all, studies [17,18]. However, we have repeated these stains on several occasions with similar results. In future studies, it would be of interest to examine other elements of Notch signaling, as set out in the introduction, in motor neurons, astroglia, oligodendroglia, and microglia, at different time points in the mSOD1 mouse.

A reduction in Shh signaling can be expected to have important consequences. Even though Shh is a developmental morphogen, several studies have shown that Shh continues to play a trophic role in the maintenance of motor neurons, especially in the face of oxidative or excitotoxic insult. The reduction in Notch signaling and the cytosolic redistribution of the Gli proteins could

Fig. 4



Representative images of immunofluorescent Gli3 staining in motor neurons of lumbar spinal cords in WT and mSOD mice. For all age groups of WT mice, Gli3 (yellow arrows) showed predominantly nuclear staining in motor neurons in the lumbar spinal cord (a, c, and e). In mSOD mice, mice at 60 days showed a Gli3 staining intensity and pattern similar to that of the age-matched WT controls (b). However, the intensity of Gli3 staining was markedly reduced in the 117 days group (d) and almost absent in the 138 days group (f). Scale bar = 50 μ m. mSOD, mutant superoxide dismutase; WT, wild type.

compromise Shh signaling, and thereby contribute toward the dysfunction and death of motor neurons in ALS.

Moreover, there is a further mechanism through which loss of Shh signaling might affect adult motor neurons. Namely, retinoic acid and Shh are essential to the terminal differentiation of motor neurons during embryological development, and are essential and sufficient to differentiate cell lines such as NSC34 and Sy5y into a mature motor neuronal phenotype. However, less well understood are the factors that maintain motor neurons in their terminally differentiated state, which might include some of the same factors leading to their differentiation. It may be that a defining insult to ALS motor neurons, explaining the common disease phenotype, is a loss of differentiated

status, such that the motor neurons do not die, at least initially, but rather cease to function normally.

Three observations lend some credence to this hypothesis. First, in some mouse models of ALS, the disease onsets at a typical age, weakness progresses as expected, and animals ultimately die or are euthanized at endpoint, also as expected. Yet, when examined at *post mortem*, many or even most motor neurons in the spinal cord are still present [19] and one assumes in these cases that the motor neurons remain viable, but nonfunctional, which would be consistent with a loss of differentiation. Second, in human disease, the earliest neuropathological features are a retraction of axon from the motor endplate, also consistent with de-differentiation [20]. Finally, in our study, Notch and Shh, two factors critical to motor

neuronal differentiation, show major changes coincident with the appearance of clinical disease.

Acknowledgements

The authors acknowledge the generosity of David Hunt, whose charitable donation to research funded these experiments.

Conflicts of interest

There are no conflicts of interest.

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