Cholestenoic acids regulate motor neuron survival via liver X receptors

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Introduction

The vertebrate CNS is composed of a wide variety of neurons that are generated following tightly regulated developmental programs. Characterization of the function and specificity of molecules working on distinct neuronal populations is thus essential in order to enhance our understanding of how such complexity is achieved in the developing brain and how it is maintained in the adult brain. One means of developmental and adult regulation is via nuclear receptors. Examples of nuclear receptors expressed in embryonic and adult brain having both a developmental role (1) and a function in the adult brain (2) are the liver X receptors (LXRs) and their ligands, which are activated by oxysterols (3, 4). Analysis of double-knockout Lxra−/−Lxrb−/− mice revealed that LXRs are required for neurogenesis during ventral midbrain (VM) development (1). Moreover, adult male Lxrb−/− show progressive accumulation of lipids in the brain and loss of spinal cord motor neurons, suggesting a neuroprotective role of LXRs and their ligands on adult motor neurons (5). Similarly, the number of islet-1+ oculomotor neurons in the developing mouse midbrain (Lxra−/−Lxrb−/−) mice, indicating a role of LXRs not only in the maintenance of...
adult motor neurons, but also in their development (1). In agreement with these findings, enzymes involved in the synthesis of cholesterol and oxysterols, such as 2,3-oxidosqualene-lanosterol cyclase, are localized in islet-1’ oculomotor neurons in the mouse VM at E11.5 (1). In addition to the above, we recently found that oxysterols and other endogenous brain LXR ligands are sufficient to regulate neurogenesis in the developing VM (1, 6). While endogenous brain LXR ligands have been identified and found to regulate the development of midbrain dopamine neurons and red nucleus neurons (6), to date, no endogenous ligand capable of regulating the survival of motor neurons in vivo has been identified. In a recent study, we found that cholesterol metabolites that had the capacity to activate LXRs can be identified in human cerebrospinal fluid (CSF) (7). In order to identify novel LXR ligands that regulate motor neuron function, we delved deeper into the human CSF sterolome and examined plasma of patients with 2 different human diseases associated with upper motor neuron degeneration, hereditary spastic paraisis type 5 (SPG5) and cerebrotendinous xanthomatosis (CTX). These diseases result from mutations in the cytochrome P450 (CYP) genes CYP7B1 and CYP27A1, respectively (8-17). The enzymes coded by these genes are responsible for 7α-hydroxylation of oxysterols and (25R,26-hydroxylation of sterols, respectively, reactions that generate further oxysterols and ultimately cholestenolic acids (Figure 1A and ref. 18). Note that we have adopted the sterol nomenclature recommended by the lipid maps consortium: 26-hydroxycholesterol (26-HC) refers to cholest-26-ene-3β,7α, diol; similarly, carboxylic acids that introduce 25R stereochemistry to the side-chain are at C-26 (19). We found that specific cholestenoic acids with a 3β-hydroxy-5-ene, but not a 3-oxo-4-ene, structure activated LXRx and LXRβ in neuronal cells, increased expression of islet-1, a transcription factor required for the development of motor neurons (20-22), and promoted the survival of islet-1’ oculomotor neurons. Moreover, these effects were abolished by knockdown or knockout of the LXR-encoding genes in zebrafish or in rodent models, respectively. In addition, we showed that patients with CTX and SPG5 are unable to synthesize normal amounts of the LXR ligand 3β,7α-dihydroxycholest-4-en-3β,25-diHCO, or via 3β,7α-dihydroxycholest-5-en-26-oic acid (7αH,3O-CA), a cholestenolic acid that we found to promote neuronal survival. This is of interest in relation to the fact that patients with SPG5 present with motor neuron degeneration and spastic paraplegia. Patients with CTX may sometimes also present with spasticity, possibly due to upper motor neuron degeneration. Our findings may have important implications for neurological diseases leading to motor neuron degeneration, since LXR ligands, as well as inhibitors of specific biosynthetic enzymes in the cholesterol acid biosynthetic and metabolic pathways, may be useful pharmaceuticals for the treatment of motor neuron disorders.

Results
Specific cholestenoic acids are abundant in human CSF. We have previously shown that cholestenoic acids are abundant in human plasma (23, 24) and that human CSF also contains specific cholestenolic acids (7). Surprisingly, in CSF, the levels of cholestenoic acids were higher than those of oxysterols. In this study, we delved deeper into the CSF sterolome, particularly into the cholestenoic acid portion, using liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS) analysis. We hereby report the exact identity of 16 oxysterols and downstream metabolites, including cholestenoic acids, found in human CSF (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI68506DS1). The most abundant of these metabolites (19.48–0.40 ng/ml; Supplemental Figure 1) were 7α-hydroxy-3-oxocholest-4-en-26-oic acid (7αH,3O-CA), 3β-hydroxycholest-5-en-26-oic acid (3β-HCA), and 2 newly identified metabolites in CSF, 3β,7α-diHCA and 3β,7β-dihydroxycholest-5-en-26-oic acid (3β,7β-diHCA). Precursors of these acids, including 26-CH and newly identified 7α,26-dihydroxycholesterol (7α,26-diHC; cholest-5-ene-3β,7α,26-triol) and 7α,26-dihydroxycholest-4-en-3-one (7α,26-diHCO), were also found, but at lower levels (0.15–0.03 ng/ml). Our results thus identified 4 novel oxysterol metabolites in human CSF that were downstream of 26-CH (Figure 1A). 26-CH is metabolized via 7α,26-diHC and 7α,26-diHCO, or via 3β,7α-diHCA and 3β,7α-dihydroxycholesterol (7αH,3O-CA). While 26-CH can cross the blood-brain barrier (BBB) and enter the brain from the circulation (25), 7αH,3O-CA traverses the BBB and is exported from the brain (26). Very low levels of 24S-hydroxycholesterol (24S-CH; cholest-5-ene-3β,24S-diol), 25-hydroxycholesterol (25-CH; cholest-5-ene-3β,25-diol), and newly identified 7α,25-dihydroxycholesterol (7α,25-diHCO; cholest-5-ene-3β,7α,25-triol) and 7α,25-dihydroxycholest-4-en-3-one (7α,25-diHCO) were also found in CSF (0.08–0.03 ng/ml).

Reduced levels of 7α-hydroxylated cholestenolic acids in CSF and plasma/serum of human patients with SPG5. SPG5 presents with upper motor neuron signs and results from mutations in CYP7B1, encoding the oxysterol 7α-hydroxylase responsible for 7α-hydroxylation of side-chain oxidized sterols that is required for extrahepatic synthesis of 7αH,3O-CA and its precursor, 3β,7α-diHCA (Figure 1A and ref. 18). In order to examine the pathogenic role of such mutations, we sought to identify alterations in oxysterol and cholestenoic acid profiles in CSF and plasma from these patients and then examine the biological activities of the altered metabolites. We first studied the CSF from 3 patients with SPG5 (see Supplemental Table 3 for patient clinical data). Elevated levels of the CYP7B1 substrates 25-CH (P < 0.10), 26-CH (P < 0.07), and 3β-HCA (P < 0.02), as well as reduced levels of its products, 3β,7α-diHCA (P < 0.03) and 7αH,3O-CA (P < 0.001), were found compared with 18 individual control subjects; similar results were found when the SPG5 patients were compared with 2 healthy carrier heterozygotes with a single mutation in CYP7B1 (Figure 1, F-I, and Supplemental Table 1). When plasma was analyzed from 9 SPG5 patients (8, 10, 14), significantly elevated 25-CH (P < 0.03), 26-CH (P < 0.001), and 3β-HCA (P < 0.02) and reduced 3β,7α-diHCA (P < 0.001) and 7αH,3O-CA (P < 0.02) were found compared with control subjects (Figure 1, B-E, and Supplemental Table 2). Similar differences were found between patient samples and 3 healthy carriers. This indicates that for these metabolites, plasma represents a good surrogate for CSF. However, while 3β,7α-diHCA and 7αH,3O-CA in the CNS is normally derived from 26-CH, that found in the circulation can be derived via either the 26-CH or the 7α-hydroxycholesterol (7α-HC; cholest-5-ene-3β,7α-diol) pathway (acidic and neutral, respectively) of bile acid biosynthesis (18). Thus, in SPG5 patients (CYP7B1 mutation), the liver-specific 7α-hydroxylase CYP7A1 (neutral pathway) accounts for the residual content of 3β,7α-diHCA and 7αH,3O-CA found in the
levels of toxic 3β-hydroxy-5-ene acids, may contribute to the progressive liver disease in these patients at an early age.

CTX is a second human disease that may present with signs of motor neuron loss. It is characterized by mutations in CYP27A1. We found that the plasma of patients with CTX was essentially devoid of 26-HC and the downstream cholestenoic acids (Figure 1, B–E, and Supplemental Table 2). As reported previously (33), elevated levels of 7α-HCO were observed (Supplemental Table 2).

The absence of cholestenoic acids in plasma indicates an inability to biosynthesize C27 acids in extrahepatic steroidogenic tissue, including the CNS.

circulation. We previously investigated the plasma oxysterol and cholestenoic acid profile of 3 infants with mutations in CYP7B1 (Supplemental Table 2) resulting in oxysterol 7α-hydroxylase deficiency (O7AHD) and neonatal liver disease (27–30), as well as SPG5 in adults (31). The first identification of CYP7B1 mutations were found in a child with severe cholestasis (32), defining a new inborn error of bile acid biosynthesis. As expected by the absence of functional CYP7B1 in these patients, we found very low plasma levels of 3β,7α-diHCA (P < 0.001; Figure 1D and Supplemental Table 2), as described above for SPG5. These patients also had considerably elevated plasma levels of 24S-HC, 25-HC, and 26-HC and high levels of hepatotoxic 3β-hydroxychol-5-en-24-oic acid (3βH-Δ5-BA) compared with SPG5 patients and controls. These findings suggest that additional factors, including increased levels of toxic 3β-hydroxy-5-ene acids, may contribute to the progressive liver disease in these patients at an early age.

CTX is a second human disease that may present with signs of motor neuron loss. It is characterized by mutations in CYP27A1. We found that the plasma of patients with CTX was essentially devoid of 26-HC and the downstream cholestenoic acids (Figure 1, B–E, and Supplemental Table 2). As reported previously (33), elevated levels of 7α-HCO plus 7α-hydroxycholest-4-en-3-one (7α-HCO) and/or 7α,12α-dihydroxycholesterol (7α,12α-diHCholesterol) were observed (Supplemental Table 2). The absence of cholestenoic acids in plasma indicates an inability to biosynthesize C27 acids in extrahepatic steroidogenic tissue, including the CNS.
In summary, our data showed specific changes in 7α-hydroxylated cholestenoic acids in plasma of CTX and SPG5 patients; we thus decided to examine their effect on neural function.

Specific cholestenoic acids are ligands to LXRα and LXRβ in neural cells. In order to gain insights into the mechanism by which alterations in cholesterol metabolism causes neurological disease, we studied whether any of the cholestenoic acids present at high levels in control human CSF and deregulated in CSF or plasma of SPG5 or CTX patients work as LXR ligands. We thus focused on 26-HC, 3β-HCA, 3β,7α-diHCA, its isomer 3β,7β-diHCA, and 7αH,3O-CA (Figure 1A) and tested their capacity to activate LXRα and LXRβ in neural cells. We previously found that 3β-HCA activates LXR (7) and here observed that 3β,7α-diHCA activates LXRα and LXRβ (Figure 2A and Supplemental Figure 2, A and B). 3β,7α-diHCA was the most potent cholestenoic acid, exhibiting 61% activity/efficacy toward LXRβ activation compared with the known LXR ligand 22R-hydroxycholesterol (22R-HC; cholest-5-ene-3β,22R-diol), followed by 3β-HCA and 3β,7β-diHCA (Supplemental Figure 2B). However 3β,7β-diHCA

**Figure 2.** Analysis of the nuclear receptor activation capacity of oxysterols and cholestenoic acids. (A) Luciferase activity in SN4741 neural cells transfected with an LXR-responsive luciferase reporter construct (LXRE) with or without LXRα and stimulated for 24 hours with 22R-HC (10 μM), a known LXRα ligand (3, 4), or the compounds indicated. (B) Similar assay in cells transfected with an FXR-responsive luciferase reporter construct (FXRE) with or without FXR and stimulated for 24 hours with CDCA, a known FXR ligand, or the compounds indicated. (C) Additional luciferase assays performed as in A with or without addition of the LXR antagonist GGPP (10 μM) along with the indicated cholesterol metabolites (10 μM each). Values in A–C are fold activation over the basal LXRE or FXRE luciferase activity (set to 1) (n = 3). *P < 0.05, **P < 0.01 vs. vehicle or as indicated by brackets, Mann-Whitney test. (D) TR-FRET LXRβ coactivator assay, used to determine the binding affinity of cholestenoic acids as well as the known LXR ligands GW3965 and 24,25-EC toward the LXRβ-LBD (n = 3). The 520:495 TR-FRET ratio was determined as described in Methods. *P < 0.05 vs. vehicle for ≥10 μM 3β,7α-diHCA, ≥10 μM 3β,7β-diHCA, ≥5 μM 24,25-EC, and ≥5 μM GW3965, Mann-Whitney test. (E) 3β,7α-diHCA, 3β,7β-diHCA and 3β-HCA induced significant increases in Abca1, Abcg1, and Srebf1 in SN4741 cells (n = 3). *P < 0.05, **P < 0.01 vs. vehicle, Mann-Whitney test.
had the lowest 50% effective concentration (EC\textsubscript{50}), followed by 3β,7α-diHCA and 3β-HCA. In addition, we confirmed in our system the capacity of 24S-HC and 25-HC to activate LXR and observed that 26-HC had no significant effect. We also tested the activation capacity of 7α,3O-CA, its 7β-isomer, 7β-hydroxy-3-oxocholest-4-en-26-oic acid (7βH,3O-CA), and the precursors 7α,26-diHCA and 7α,26-diHCO, none of which showed significant activity (Figure 2A and Supplemental Figure 2A). Moreover, in order to examine whether the identified acidic ligands exert their effect by binding to LXR, we used the LXR antagonist geranylgeranyl pyrophosphate (GGPP; ref. 34), which blocked their activity (Figure 2C), indicating that the acids are indeed LXR ligands. The activity of the known LXR ligands 22R-HC and 25-HC was similarly blocked. In order to confirm that the cholestenoic acids are indeed agonists to LXR, we tested the LXR

3β,7β-diHCA, or 3β,7β-diHCA, and 3β,7β-diHCA, or 3β,7β-HCA, increased islet-1–GFP expression, but embryonic toxic effect that impaired survival and precluded further in vitro, we next sought to identify their effect in vivo. In particular, we focused on the expression of islet-1, a transcription factor expressed in all postmitotic motor neurons (20, 37) and required for multiple aspects of motor neuron development, including motor neuron specification, motor column formation, axonal growth, and maintenance of spinal motor neuron identity (38). We used transgenic zebrafish embryos expressing GFP driven by the isl1 gene promoter/enhancer sequences (Tg isl1:GFP) (39, 40) to screen for biologically active compounds in vivo. Previous studies have indicated that islet-1 protein is required for the formation of zebrafish primary motor neurons and is conserved throughout vertebrate evolution (21). Treatment of Tg isl1:GFP embryos with the weak LXR ligands 3β-HCA and 3β,7β-diHCA, as well as CDCA, the most potent FXR ligand, had a deleterious toxic effect that impaired survival and precluded further in vivo analysis. Interestingly, we found that 2 LXR agonists, 3β,7α-diHCA and 3βH,7O-CA, increased islet-1–GFP expression, but had no significant effect on the number of islet-1 cells, in the different cranial nerves examined (loci III, IV, V, VII, and X; Figure 3, A and B). These effects were specific, as 7αH,3O-CA, which is not an LXR ligand, failed to regulate islet-1–GFP expression (Figure 3B). Notably, the increase in islet-1–GFP expression observed in response to the specific acidic compounds was evident in all cranial nerves studied (Figure 3B). Importantly, the increased islet-1–GFP expression in response to 3β,7α-diHCA and 3βH,7O-CA was also accompanied by an increased level of isl1 mRNA, as assessed by quantitative PCR (Figure 3C). To further verify our results, we examined whether these cholestenoic acids regulated the in vivo expression of endogenous LXR target genes, such as abca1. Our results showed enhanced expression of abca1 by both 3β,7α-diHCA and 3βH,7O-CA, but not by 7αH,3O-CA (Figure 3D). Finally, in order to determine whether these effects are actually mediated by LXRs, we performed lxr morpholino (MO) injections in Tg isl1:GFP zebrafish. Interestingly, we found that lxr MO injections abolished the in vivo increase in islet-1–GFP levels by 3β,7α-diHCA and 3βH,7O-CA, compared with control scrambled MO (Supplemental Figure 4). Thus, our data showed that cholestenoic acids are capable of activating endogenous LXR target genes in vivo and regulate the in vivo expression of islet-1 in brain motor neurons via LXR.

3β,7α-diHCA and 3βH,7O-CA increase islet-1 expression in islet-1–GFp zebrafish embryos. Having established that cholestenoic acids that are altered in CTX or SPG5 can activate LXRs in vitro, we next sought to identify their effect in vivo. In particular, we focused on the expression of islet-1, a transcription factor expressed in all postmitotic motor neurons (20, 37) and required for multiple aspects of motor neuron development, including motor neuron specification, motor column formation, axonal growth, and maintenance of spinal motor neuron identity (38). We used transgenic zebrafish embryos expressing GFP driven by the isl1 gene promoter/enhancer sequences (Tg isl1:GFP) (39, 40) to screen for biologically active compounds in vivo. Previous studies have indicated that islet-1 protein is required for the formation of zebrafish primary motor neurons and is conserved throughout vertebrate evolution (21). Treatment of Tg isl1:GFP embryos with the weak LXR ligands 3β-HCA and 3β,7β-diHCA, as well as CDCA, the most potent FXR ligand, had a deleterious toxic effect that impaired survival and precluded further in vivo analysis. Interestingly, we found that 2 LXR agonists, 3β,7α-diHCA and 3βH,7O-CA, increased islet-1–GFP expression, but had no significant effect on the number of islet-1 cells, in the different cranial nerves examined (loci III, IV, V, VII, and X; Figure 3, A and B). These effects were specific, as 7αH,3O-CA, which is not an LXR ligand, failed to regulate islet-1–GFP expression (Figure 3B). Notably, the increase in islet-1–GFP expression observed in response to the specific acidic compounds was evident in all cranial nerves studied (Figure 3B). Importantly, the increased islet-1–GFP expression in response to 3β,7α-diHCA and 3βH,7O-CA was also accompanied by an increased level of isl1 mRNA, as assessed by quantitative PCR (Figure 3C). To further verify our results, we examined whether these cholestenoic acids regulated the in vivo expression of endogenous LXR target genes, such as abca1. Our results showed enhanced expression of abca1 by both 3β,7α-diHCA and 3βH,7O-CA, but not by 7αH,3O-CA (Figure 3D). Finally, in order to determine whether these effects are actually mediated by LXRs, we performed lxr morpholino (MO) injections in Tg isl1:GFP zebrafish. Interestingly, we found that lxr MO injections abolished the in vivo increase in islet-1–GFP levels by 3β,7α-diHCA and 3βH,7O-CA, compared with control scrambled MO (Supplemental Figure 4). Thus, our data showed that cholestenoic acids are capable of activating endogenous LXR target genes in vivo and regulate the in vivo expression of islet-1 in brain motor neurons via LXR.
tions of 3β,7α-diHCA and 3β,7β,7O-CA increased islet-1+ oculomotor cell numbers (Figure 4A). EC50 values were 5.21 μM for 3β,7α-diHCA, and IC50 values were 4.44 μM for 3μdiHCA and 3.58 μM for 3β,7α-diHCA and 3β,7β,7O-CA. Interestingly, the effects of 3β,7α-diHCA and 3β,7O-CA were specific, as they did not affect the number of other midbrain neurons, such as tyrosine hydroxylase–positive (TH+) dopamine neurons, GABAergic neurons, or red nucleus neurons in the cultures (data not shown). In order to examine whether the effects of 3β,7α-diHCA and 3β,7O-CA were specifically mediated by LXR receptors in the rodent brain, we performed progenitor brain cultures from Lxrα−/−Lxrb−/− mice. Remarkably, the effects of 3β,7α-diHCA and 3β,7O-CA (at their most efficient concentration) on islet-1+ neurons were eliminated (Figure 4C), confirming that they regulate the number of islet-1+ cells in the rodent brain through LXRs. Moreover, the effects of these acids were blocked by GGPP (Figure 4D), further suggesting that the observed effects were mediated by LXR receptors.

Interestingly, 26-HC, a precursor of cholestenoic acids in the acidic pathway of bile acid biosynthesis (18) that had no LXR activational effect in luciferase assays in neural cells (Figure 2 and ref. 6), also exhibited no effect on the number of islet-1+ cells (data not shown). Furthermore, the effects of 3β,7α-diHCA, 3β,7β-diHCA, and 3β,7O-CA on islet-1+ cells were not altered by the known LXR ligand 22R-HC (Supplemental Figure 3C), which does not show an effect toward islet-1+ cells (data not shown). Arrows indicate loci III, IV, V, VII, and X (37), which are evolutionarily homologous to the cranial nerves in humans (40). Locus III contains the oculomotor neurons, locus IV the trochlear neurons, locus V the trigeminal motor neurons, locus VII the facial motor neurons, and locus X the cell bodies of the vagus nerve. Scale bar: 50 μm. (B) Quantification of islet-1–GFP signal intensity in the different loci (n = 4). 24,25-EC was used as a positive control. (C) and (D) mRNA levels of (C) isl1 and (D) abcb1 after treatment with the indicated compounds. Data are mean ± SEM (n = 3). *P < 0.05, **P < 0.01 vs. respective vehicle, Mann-Whitney test.

Figure 3. Effects of cholestenoic acids on zebrafish motor neurons. Tg[isl1:GFP] embryos were incubated with 10 μM test compound or vehicle added to medium, and the medium was replaced every 12 hours with fresh solution (containing test compound or vehicle). Immunocytochemistry was performed using an anti-GFP antibody at 48 hpf. (A) Dorsal (top) and dorsolateral (bottom) views of the head/upper back region of embryos treated with vehicle, 3β,7α-diHCA, or 3β,7O-CA. Arrows indicate loci III, IV, V, VII, and X (37), which are evolutionarily homologous to the cranial nerves in humans (40). Locus III contains the oculomotor neurons, locus IV the trochlear neurons, locus V the trigeminal motor neurons, locus VII the facial motor neurons, and locus X the cell bodies of the vagus nerve. Scale bar: 50 μm. (B) Quantification of islet-1–GFP signal intensity in the different loci (n = 4). 24,25-EC was used as a positive control. (C) and (D) mRNA levels of (C) isl1 and (D) abcb1 after treatment with the indicated compounds. Data are mean ± SEM (n = 3). *P < 0.05, **P < 0.01 vs. respective vehicle, Mann-Whitney test.
Figure 4. Specific cholestenoic acids increase the number of islet-1+ oculomotor neurons, promote neuronal survival, or are toxic in mouse E11.5 brain primary cultures. (A) Dose-response curves for quantification of islet-1+ cells in mouse E11.5 brain primary cultures from WT embryos treated with the indicated compounds. \( ^* P < 0.05 \) vs. vehicle for ≥5 μM 3β,7α-diHCA, ≥10 μM 3β,7O-CA, ≥2 μM 3β,7β-diHCA, and ≥2 μM 3β-HCA, Mann-Whitney test. (B) Representative islet-1- and NKX6.1-stained cell nuclei treated with 3β,7α-diHCA (10 μM), 3β,7O-CA (10 μM), 3β,7β-diHCA (2 μM), or 3β-HCA (2 μM). (C) Quantification of islet-1+ cells in primary cultures from WT or Lxrα--/--Lxrβ--/-- embryos treated with 3β,7α-diHCA or 3β,7O-CA (10 μM). \( ^* P < 0.05 \) vs. vehicle, Mann-Whitney test. (D) Effect of 10 μM GGPP on treatment in the WT group. \( ^* P < 0.05 \), Mann-Whitney test. (E) Dose–response curves for quantification of active caspase-3+ cells in mouse E11.5 brain primary cultures. Very high cell death in the cultures is denoted by ¶. \( ^* P < 0.05 \) vs. vehicle for ≥10 μM 3β,7α-diHCA, ≥2 μM 3β,7β-diHCA, and ≥2 μM 3β-HCA, Mann-Whitney test. (F) Representative active caspase-3- and islet-1-stained cell nuclei treated as in B. (G) Quantification of active caspase-3+ cells in primary cultures (WT group) treated as indicated, with or without 10 μM GGPP. \( ^* P < 0.05 \) vs. vehicle or as indicated by brackets, Mann-Whitney test. All data are mean ± SEM (n = 3). Scale bars: 20 μm.
ber of BrdU+ cells in the cultures (Supplemental Figure 6B), which suggests that they do not modulate proliferation. Finally, we tested whether cholestenoic acids regulate neuronal survival, as assessed by active caspase-3 staining to detect the number of cells undergoing apoptosis in the cultures. Interestingly, treatment with 3β,7α-diHCA rescued the toxic effect of 2 μM 3β,7β-diHCA or 3β-HCA on islet-1+ cells and reduced neuronal cell death induced by these acids, as indicated by the number of active caspase-3+ cells, in mouse E11.5 brain primary cultures. *P < 0.05, **P < 0.01, Mann-Whitney test. (F) Mouse embryos were injected with vehicle or cholestenoic acid into the mesencephalic ventricle in utero at E11.5 and collected at E13.5. (G–N) Midbrain coronal sections showing islet-1+ motor neurons (green) and neighboring TH+ neurons (red) (G–J), and higher magnification of islet-1+ cells (K–N), for embryos injected with vehicle (G and K), 5 μM 3β,7α-diHCA (H and L), 1 μM 3β-HCA (I and M), and 1 μM 3β-HCA plus 5 μM 3β,7α-diHCA (J and N). (O) Quantification of islet-1+ cell number in each group. *P < 0.05, **P < 0.01, 1-way ANOVA with LSD post-hoc test. Scale bars: 100 μm (G–J); 20 μm (K–N). All data are mean ± SEM (n = 3).

Figure 5. Competition between cholestenoic acid effects, and 3β,7α-diHCA promotes motor neuron survival in vivo. (A–C) TR-FRET LXRβ coactivator assays. (A) The effect of 10 μM 3β,7α-diHCA was dose-dependently competed by increasing concentrations (incr.) of 3β,7β-diHCA and 3β-HCA (both *P < 0.05 for ≥2 μM). Similarly, (B) the effect of 10 μM 24,25-EC was dose-dependently competed by 3β,7α-diHCA (*P < 0.05 for ≥10 μM), 3β,7β-diHCA (*P < 0.05 for ≥10 μM), and 3β-HCA (*P < 0.05 for ≥5 μM), and (C) the effects of 3β,7α-diHCA, 3β-HCA, 3β,7β-diHCA, and 24,25-EC (all 10 μM) were dose-dependently competed by GGPP (all *P < 0.05 vs. respective no-GGPP group). Data were analyzed by Mann-Whitney test. (D and E) 10 μM 3β,7α-diHCA rescued the toxic effect of 2 μM 3β,7β-diHCA or 3β-HCA on islet-1+ cells and reduced neuronal cell death induced by these acids, as indicated by the number of active caspase-3+ cells, in mouse E11.5 brain primary cultures. *P < 0.05, **P < 0.01, Mann-Whitney test. (F) Mouse embryos were injected with vehicle or cholestenoic acid into the mesencephalic ventricle in utero at E11.5 and collected at E13.5. (G–N) Midbrain coronal sections showing islet-1+ motor neurons (green) and neighboring TH+ neurons (red) (G–J), and higher magnification of islet-1+ cells (K–N), for embryos injected with vehicle (G and K), 5 μM 3β,7α-diHCA (H and L), 1 μM 3β-HCA (I and M), and 1 μM 3β-HCA plus 5 μM 3β,7α-diHCA (J and N). (O) Quantification of islet-1+ cell number in each group. *P < 0.05, **P < 0.01, 1-way ANOVA with LSD post-hoc test. Scale bars: 100 μm (G–J); 20 μm (K–N). All data are mean ± SEM (n = 3).
βH,7O-CA, we attribute the increase in islet-1+ oculomotor neurons, indicative of a selective survival effect of 3β,7β-dihCA on oculomotor neurons in vivo. Upon injection of 3β-HCA, the number of islet-1+ oculomotor neurons was reduced, showing the toxic effect of this metabolite on motor neurons in vivo. Finally, coinjection of 3β-HCA and 3β,7α-dihCA reversed the loss of oculomotor neurons induced by 3β-HCA, indicating that 3β,7α-dihCA protects oculomotor neurons against cholestenoic acids such as 3β-HCA in vivo. These data further confirm the specificity of the neuronal survival and toxic effects of these cholestenoic acids on islet-1+ neurons, but not TH+ neurons, as well as the competitive interaction between prosurvival and toxic cholestenoic acids on motor neurons in vivo.

In summary, our results indicate that cholestenoic acids regulate the number of islet-1+ motor neurons by controlling islet-1 expression (3βH,7O-CA) and competitively regulating neuronal survival in a positive (3β,7α-dihCA) or negative (3β,7β-dihCA and 3β-HCA) manner.

Discussion
The results presented herein show that cholestenoic acids are not mere intermediate metabolites of bile acid biosynthesis, but rather represent a diverse family of bioactive compounds capable of regulating nuclear receptor function. Cholestenoic acids were found to specifically activate LXR and elicit an exquisite array of functions, ranging from the regulation of islet-1 expression to the positive and negative regulation of motor neuron survival both in vitro and in vivo. Moreover, our findings identified cholestenoic acids in human CSF to be deregulated in plasma of CTX and SPG5 patients. Importantly, an absence of neuroprotective cholestenoic acids was found in CTX patient plasma, while a combination of decreased neuroprotective and increased toxic cholestenoic acids was detected in SPG5 plasma. These results are consistent with cholestenoic acids being key regulators of motor neuron function in development and disease.

Cholesterol is present at high levels in the CNS of vertebrates and is metabolized in brain predominantly to 24S-HC (42), which accounts for about two-thirds of brain cholesterol metabolism (43). Low levels of 26-HC have been found in human and mouse brain (Supplemental Results and Supplemental Table 4) and in human CSF, where it may be imported from the blood (25, 44–46). Conversely, 7αH,30-CA, a metabolic product of 26-HC, is exported from brain to blood in humans (26). In the current study, we found 4 intermediates in the biosynthesis of 7αH,30-CA from 26-HC via CYP27A1 and CYP7B1 to be present in normal CSF: 3β-HCA, 3β,7α-dihCA, 7α,26-dihIC, and 7α,26-dihOC (Figure 1A). While 3β-HCA and 7α,26-dihIC were previously found in human neu-
ral tissue (46, 47), 3β,7α-diHCA and 7α,26-diHCO have not been previously found in neural tissue or CSF. Importantly, the identification of these intermediates in the biosynthesis of 7αH,3O-CA lends further support to the hypothesis that 7αH,3O-CA is biosynthesized in the human brain (26). This pathway is also conserved in rodents, in which 26-HC is the precursor for the synthesis of 3β-HCA in fetal neurons and of 3β,7α-diHCA and 7αH,3O-CA in fetal astrocytes (Figure 1A and ref. 48). There have been few studies of specific brain regions for cholestenoic acids; however, human retina has been found to contain 3β-HCA at a level of up to 130 pmol/mg protein, about 10% of the level of 24S-HC in brain gray matter (47), whereas in chronic subdural hematoma, 7αH,3O-CA has been found at a level of 1.5 μM (49). Future studies will be directed at profiling cholestenoic acids in distinct brain regions.

Surprisingly, αH,3O-130 pmol/mg protein, about 10% of the level of 24S-HC in brain human retina has been found to contain 3β, 7α, 7β diHCA and of 3β,7α-diHCA in fetal neurons and of 3β,7α-diHCA in rodent models (Figure 1A and ref. 48). There have been few studies of specific brain regions for cholestenoic acids; however, human retina has been found to contain 3β-HCA at a level of up to 130 pmol/mg protein, about 10% of the level of 24S-HC in brain gray matter (47), whereas in chronic subdural hematoma, 7αH,3O-CA has been found at a level of 1.5 μM (49). Future studies will be directed at profiling cholestenoic acids in distinct brain regions.

Numerous studies in recent years have linked LXR to neuronal degeneration (2, 5, 51–53). These studies have used Lxra–/– and Lxrb–/– mice. Indeed, both LXR isoforms are expressed in brain (1, 54), and the knockout mice show progressive lipid accumulation in brain, abnormal BBB, increased reactive microglia, astrogliosis, and degeneration of adult spinal cord motor neurons (2, 5). Interestingly, a decrease in the number of oculomotor neurons was also detected during development in Lxra–/–Lxrb–/– mice at E11.5 (1). However, the identity of endogenous brain LXR ligands that regulate motor neuron function was unknown. Here we used zebrafish to study the in vivo function of acidic LXR ligands newly identified in human CSF (i.e., 3β,7α-diHCA and 3β,7β-diHCA), and the 7-oxo intermediate in their epimerization reaction (3βH,7O-CA) are all LXR ligands effective at micromolar concentrations. Moreover, none of these acids were found to activate FXR, VDR, or NURR1 in neural cells, thereby confirming the specificity of their effect on LXR.

In summary, we identified 3β,7α-diHCA and 3β,7β-diHCA as LXR ligands present in human CSF. Of these, 3β,7α-diHCA and the previously identified LXR ligand 3β-HCA were found to cause cell death, and the latter was present at high levels in patients with SPG5. Instead, 3β,7α-diHCA, which was found at low levels in...
SPG5 and absent in CTX patients, promoted motor neuron survival, while 3βH,7α-CA regulated islet-1 expression levels. Thus, our results uncovered several novel functions of cholestenoic acids and identified them as LXR ligands and key regulators of motor neuron function in development and disease. We suggest that factors with neuroprotective function, such as 3β,7α-dihCA, may thus find a therapeutic application to prevent motor neuron loss. This suggestion is supported by our finding that 3β,7α-dihCA, a cholestenoic acid that decreased in SPG5, prevented cell loss by 3β-HCA, a cholestenoic acid that accumulates in SPG5.

**Methods**

**Extraction of sterols.** Sterols were extracted from CSF, plasma, or mouse brain into ethanol and fractionated by reversed phase solid phase extraction (SPE) to give a cholestenoic acid– and oxysterol-rich fraction devoid of cholesterol (7, 55, 56).

**Charge-tagging of sterols.** The sterols were charge-tagged with the GP-hydrazone as described previously (7, 55, 56). This greatly enhances their response when analyzed by LC-ESI-MS and tandem mass spectrometry (MS²).

**Reagents.** HPLC-grade water and solvents were from Fisher Scientific or Sigma-Aldrich. Authentic sterols, steroids, cholestenoic acids, bile acids, and their precursors were from Avanti Polar Lipids, Steraloids, Sigma-Aldrich, or from previous studies in our laboratories. Girard P (GP) reagent [1-(carboxymethyl)pyridinium chloride hydrate] was from TCI Europe or synthesized in earlier studies, and cholesterol was obtained via natural spawning and staged in hours postfertilization (hpf) or days post fertilization, according to Kimmel et al. (57). Embryos older than 24 hpf were treated with 0.03% phenylthiourea (PTU) to inhibit pigmentation. MO injections were performed using splice site–specific zebrafish lsr MO, as previously reported (58). MO-injected embryos were immediately dechorionated, transferred to a 96-well plate, and exposed to DMSO- or ligand-treated medium. Tg isl1-GFP] embryos were collected by natural mating, immediately dechorionated (at the 1-cell stage), and transferred to a 96-well plate. Each compound tested was obtained as 10 mM stock and diluted in embryo medium to a final concentration of 10 μM, and 200 μl was added to each well. DMSO- or propan-2-ol–treated embryo medium was used as control. Ligand solutions were replaced every 12 hours with fresh ligand solution prepared in PTU-treated embryo medium. Embryos were collected at 48 hpf, fixed for 4 hours at room temperature with 4% paraformaldehyde (PFA), and then washed and kept in PBST. Immunocytochemistry was performed using an anti-GFP antibody, and fluorescence was viewed and photographed using a Zeiss Axioplan compound microscope and a Zeiss Axioacam digital camera.

**Quantitative PCR.** Total RNA was extracted from SN4741 cells and from zebrafish treated with the compounds of interest using the RNeasy Mini Kit (Qiagen); 1 μg was treated with RQ1 RNase-free DNase (Promega) and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) and random primers (Invitrogen) (RT+ reaction). Parallel reactions without reverse transcriptase enzyme were performed as a control (RT– reaction), and Sybergreen real-time quantitative PCR assays were carried out. Expression levels were obtained by normalization to the value of the housekeeping gene encoding actin or 18S, obtained for every sample in parallel assays.

**Mice.** Mice were housed, bred, and treated according to the guidelines of the European Communities Council (directive 86/609/EEC) and the Society for Neuroscience. Lxra<sup>−/−</sup>Lxrb<sup>−/−</sup> mouse cell cultures were from the colony at the Department of Biosciences and Nutrition at Novum, Karolinska Institutet. Male and female WT and Lxra<sup>−/−</sup>Lxrb<sup>−/−</sup> mice were generated as previously described (59, 60). Mice were backcrossed onto a C57BL/6 background for 10 generations. Male Cyp7b1<sup>−/−</sup> mouse brain and plasma were from animals generated at the University of Edinburgh. Male mice homozygous for targeted disruption of the Cyp7b1 gene (61) congenic on the C57BL/6 background (>15 generations backcrossed to C57BL/6) were generated and treated according to the guidelines of the European Communities Council (directive 86/609/EEC) and the Society for Neuroscience. Lxra<sup>−/−</sup>Lxrb<sup>−/−</sup> WT littermate controls were generated from Cyp7b1<sup>−/−</sup> crosses. Male Cyp27a1<sup>−/−</sup> mouse tissue and plasma was purchased from The Jackson Laboratory (strain B6.129-Cyp27a1m1(Stj/)) ref. 62). The Cyp27a1<sup>−/−</sup> colony was backcrossed to C57BL/6J inbred mice for approximately 4 generations.
12 generations by the donating investigator (62) prior to sending to The Jackson Laboratory Repository. Upon arrival, mice were bred to C57BL/6J inbred mice for at least 1 generation to establish the colony. WT animals from the colony were used as controls. Tissue and blood sampling from these mice was performed under the aegis of the UK Scientific Procedures (Animals) Act, 1986.

**Primary midbrain cultures.** Brains from E11.5 mice were obtained, and the midbrain region was dissected, mechanically dissociated, plated on poly-D-lysine (150,000 cells/cm²), and grown in serum-free N2 media consisting of F12/DMEM (1:1 mixture) with 10 ng/ml insulin, 100 μg/ml apo-transferrin, 100 μM putrescine, 20 nM progesterone, 30 nM selenium, 6 mg/ml glucose, and 1 mg/ml BSA. Cells were treated for 3 days in vitro with the compounds of interest, fixed with 4% PFA, and processed for staining using appropriate antibodies.

For BrdU analysis, cells were treated with BrdU 1 hour after plating, and media was replaced with fresh medium after 16 hours. After a further 2 days in culture, cells were treated for 30 minutes with 2N HCl, and immunocytochemistry was performed to evaluate the number of double-positive BrdU+/islet-1+ cells (a measure of motor neuron neurogenesis). Hoechst staining was performed by permeabilizing cells with a 0.3% Triton-X 100/PBS solution for 5 minutes followed by incubation with Hoechst 33258 (Sigma-Aldrich) for 10 minutes.

**Immunocytochemistry.** Cells were fixed in 4% PFA, washed in PBS, and blocked in 5% normal goat serum/PBS for 1 hour at room temperature. Primary antibodies were diluted in PBS (pH 7.4), 0.3% Triton X-100, and 1% BSA, and incubations were carried out overnight at 4°C or at room temperature for 2 hours. The antibodies used were anti-BrdU (diluted 1:400; Abcam), anti-islet-1 (1:100; Developmental Studies Hybridoma Bank), anti–cleaved caspase-3 (Asp175) (1:100; Cell Signaling Technology), anti-TH (1:1,000; Pel-Freeze), anti-GABA (1:1,000; Sigma-Aldrich), anti–BRN3A (1:250; Millipore), anti-NKX6.1 (1:200; Novus Biologicals), anti-ChAT (1:500; Millipore), and appropriate secondary antibodies (Jackson ImmunoResearch or Alexa). Cells positive for the corresponding marker were counted directly at the microscope at ×20 magnification. Cells were counted in every well — in 8 consecutive fields (from one side of the well to the other, passing through the center) — in 3 different wells per experiment and 3 different experiments per condition. Random images of the wells were taken for every condition to document the result, and representative images were subsequently selected to represent the quantitative data. Photos were acquired with a Zeiss Axioplan microscope and a Hamamatsu camera C4742-95 using Openlab software.

**Intraventricular injections in utero.** Female WT CD-1 mice (25–35 g; Charles River Breeding Laboratories) were used for these experiments. For embryo analyses, WT CD-1 mice were mated overnight, and noon of the day the plug was considered E0.5. E11.5 pregnant females were deeply anesthetized using isoﬂurane (IsoFlo; Abbott Labs), and the uterine horns were accessed through an abdominal incision. 1 μl 3μg-dihCA (5 μM) or 3μg-hCA (1 μM) or vehicle solution (isopropanol; 50% v/v) was injected into the cerebral aqueduct. The uterine horns were replaced into the abdominal cavity, which was then closed with sutures. Embryos were analyzed 48 hours later.

**Immunohistochemical analysis of rodent embryos.** Embryos were dissected out of the uterine horns in ice-cold PBS, fixed in 4% PFA for 4 hours to overnight, cryoprotected in 15%-30% sucrose, frozen in Tissue-Tek OCT compound (Sakura Finetek) on dry ice, and stored at −80°C until use. Serial coronal 14-μm sections of the brain were obtained on a cryostat. 14-μm serial coronal sections through the midbrain region were cut on a cryostat and placed serially on 10 slides. Slides 1 and 6 were subjected to immunohistochemistry. Sections were preincubated for 1 hour in blocking solution followed by incubation at 4°C overnight with the following primary antibodies: anti-TH (1:750, Pel-Freeze), anti-islet-1 (1:100; Developmental Studies Hybridoma Bank), and anti–cleaved caspase-3 (Asp175) (1:100; Cell Signaling Technology). After washing, slides were incubated for 1–2 hours at room temperature with the appropriate fluorophore-conjugated (Cy2, Cy3, and Cy5, 1:300, Jackson Laboratories; Alexa Fluor 488, Alexa Fluor 555, and Alexa Fluor 647, 1:1,000, Invitrogen) secondary antibodies. Confocal images were taken with a Zeiss LSM5 Exciter or LSM700 microscope.

**Statistics.** Statistical analyses (Mann-Whitney test, Dunnett T3 test, and 1-way ANOVA with LSD post-hoc test) were performed using Prism 4 (GraphPad Software). All data represent mean ± SEM. A P value less than 0.05 was considered significant.

**Study approval.** Human samples, collected according to the principles of the Declaration of Helsinki, were provided to University Hospital Basel, Barts Health NHS Trust, St. Mary’s Hospital, Institute of Child Health, Conegliano Research Centre, Federico II University, University of Tubingen, Kurume University School of Medicine, and University Hospital “Attikon” with written informed consent and IRB and ethical approval. Ethical approval was granted by Stockholm Norra Djurförsöksstätts Nämnd for zebrafish experimentation (nos. N293/09 and N338/10), mouse experimentation (nos. N154/06, N145/09, N370/09, and N273/11), and in utero experimentation (no. N486/12).

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