Histone acetylation as a new mechanism for bilirubin-induced encephalopathy in the Gunn rat

Eleonora Vianello1, Stefania Zampieri2, Thomas Marcuzzo3, Fabio Tordini4,5, Cristina Bottin3, Andrea Dardis2, Fabrizio Zanconati3, Claudio Tiribelli4 & Silvia Gazzin1

Bilirubin neurotoxicity has been studied for decades and has been shown to affect various mechanisms via significant modulation of gene expression. This suggests that vital regulatory mechanisms of gene expression, such as epigenetic mechanisms, could play a role in bilirubin neurotoxicity. Histone acetylation has recently received attention in the CNS due to its role in gene modulation for numerous biological processes, such as synaptic plasticity, learning, memory, development and differentiation. Aberrant epigenetic regulation of gene expression in psychiatric and neurodegenerative disorders has also been described. In this work, we followed the levels of histone 3 lysine 14 acetylation (H3K14Ac) in the cerebellum (CII) of the developing (2, 9, 17 days after the birth) and adult Gunn rat, the natural model for neonatal hyperbilirubinemia and kernicterus. We observed an age-specific alteration of the H3K14Ac in the hyperbilirubinemic animals. The GeneOntology analysis of the H3K14Ac linked chromatin revealed that almost 45% of H3K14Ac ChiP-Seq TSS-promoter genes were involved in CNS development including maturation and differentiation, morphogenesis, dendritogenesis, and migration. These data suggest that the hallmark CII hypoplasia in the Gunn rat occurs also via epigenetically controlled mechanisms during the maturation of this brain structure, unraveling a novel aspect of the bilirubin-induced neurotoxicity.

Bilirubin toxicity to the CNS has been extensively studied for decades and has been shown to be linked to the activation of multiple complex signal cascades, and affects potential toxic/adaptation mechanisms in the brain through gene expression modulation. Examples include oxidative stress and the antioxidant response, excitotoxicity, inflammation, intracellular trafficking, protein degradation, apoptosis, as well as bilirubin transport and bilirubin oxidation (reviewed in1). Epigenetic processes, such as histone acetylation and DNA methylation, regulate the expression of genes through modifications of DNA structure and accessibility. These regulatory mechanisms often contribute to the onset and progression of human neurological disorders, and are altered by toxic compounds (e.g.: cocaine, alcohol)2–4. Indeed, histone acetylation is considered an integral part of brain development and differentiation, synaptic plasticity, learning, memory, and neuron maintenance and survival5–7. Notably, it is reported that temporal changes in gene expression by acetylation/deacetylation of gene promoters induce persistent changes in the cell (e.g. cell fate), changes in the neurological behaviour5, as well induction of excitotoxicity, calcium overload, oxidative stress, inflammation and apoptosis8, with the last five described mechanisms in hyperbilirubinemic animals and humans. This suggests the possibility of a link between the hyperbilirubinemic phenotype and the epigenetic. On this basis, we decided to investigate the effect of hyperbilirubinemia on the epigenetic control of the CII hypoplasia.

1Fondazione Italiana Fegato-Onlus, Bldg. Q, AREA Science Park, ss14, Km 163.5, Basovizza, 34149, Trieste, Italy.
2University Hospital Santa Maria della Misericordia, Udine. P.le Santa Maria della Misericordia 15, 33100, Udine, Italy.
3Department of Medical Sciences, Ospedale di Cattinara, Università degli Studi di Trieste, Strada di Fiume 447, 34149, Trieste, Italy.
4Cancer Genomics Laboratory, Fondazione Edo ed Elvo Tempia Valenta, Via Malta 3, 13900, Biella, Italy.
5Computer Science Department, University of Torino, 10100, Torino, Italy. Correspondence and requests for materials should be addressed to S.G. (email: silvia.gazzin@fegato.it)

Received: 14 March 2018
Accepted: 31 August 2018
Published online: 12 September 2018
Results

Serum bilirubin and cerebellar development. To evaluate the possible correlation between serum bilirubin and the levels of H3K14Ac, we quantified total and free bilirubin in the blood, and the Cll weight in hyperbilirubinemic pups (jj) and normobilirubinemic littermates (control: ctrl) from 2 days after birth (P2) until the adult age. At every post-natal age, the total serum bilirubin (TSB, Fig. 1A) was statistically higher in jj animals compared to ctrl (Σ8.5 lifelong, one-way ANOVA: \( p \leq 0.0001 \), followed by Tukey post-test, \( p \leq 0.001 \)). At P2, the TSB was about of 190\( \mu M \), peaking at P17 (Σ256\( \mu M \)), and stabilizing in the adulthood (Σ126\( \mu M \)), (ever significantly higher than in ctrl, one-way ANOVA: \( p \leq 0.0001 \), followed by Tukey post-test, \( p \leq 0.001 \)).

Free bilirubin is the moiety able to cross the blood-brain interfaces leading to neurological damage. In the absence of a reliable method for a direct quantification in the rat, free bilirubin was calculated as previously

Figure 1. Total Serum Bilirubin (TSB), calculated free bilirubin (cBf) in the blood, cerebellar weight, and Western blot analysis of the level of histone 3 acetylation (H3K14Ac) P: post-natal age in days, Adult: more than 1-year-old. Black bars jj rats, White bars: ctrls. (A) TSB (\( \mu M \)); (B) cBf (nM), (C) Cll weight (mg/animal). Results are expressed as mean ± S.D. of 6–15 animals each group and post-natal age. One way ANOVA followed by Tukey post-test: ***\( p < 0.001 \). (D) H3K14Ac levels in the Cll of jj animals vs. ctrl. Results are as mean ± S.D. of 3–6 animals each group and post-natal age. Unpaired t-test with Welch correction, *\( p < 0.05 \) vs. age matched ctrl.
described\textsuperscript{14}. Differently from TSB, the calculated Bf (cBf, Fig. 1B) level in jj pups dropped during development (P2 $\Sigma$150 nM, P9 $\Sigma$120 nM, P17 $\Sigma$35 nM, ever significantly higher than in ctrl, one-way ANOVA: $p < 0.0001$, followed by Tukey post-test, $p < 0.001$), falling to the levels not statistically different from those in ctrl in the adult age (adult jj $\Sigma$7 nM; One way ANOVA, followed by Tukey post-test, $p > 0.05$).

CII weight (Fig. 1C) was similar in jj and ctrl littersmates up to P9, becoming significantly different at P17 ($\Sigma30\%$ weight loss vs. age-matched ctrl, one way ANOVA followed by Tukey post-test: $p < 0.001$), and increasing later on (Adult: $\Sigma40\%$, one way ANOVA followed by Tukey post-test: $p < 0.001$).

**Western blot analysis of global acetylation of histone H3K14Ac.** The follow the level of H3K14Ac in the developing cerebellum of jj and controls rats by Western blot, we used the 07-353 anti-H3K13Ac antibody. At P2, no significant difference was observed in the level of H3K14Ac in the CII of jj animals compared to age-matched ctrl (Fig. 1D) (unpaired t-test with Welch correction, $p = 0.2687$). The level of H3K14Ac in jj was significantly increased (1.65 $\pm 0.54$ fold, unpaired t-test with Welch correction, $p < 0.0222$) at P9 and significantly decreased at P17 (0.67 $\pm 0.18$ fold, unpaired t-test with Welch correction, $p < 0.0187$). In the adults there was no difference in the level of H3K14Ac between jj and ctrl (unpaired t-test with Welch correction, $p = 0.4508$).

**ChIP-Seq analysis.** To link the effect of hyperbilirubinemia on H3K14Ac with the genes controlled by this epigenetic mechanism, the 07–353 anti-H3K13Ac antibody used for Western blot analysis was also used to perform chromatin immunoprecipitation, followed by DNA sequencing (ChIP-Seq – full result available on GEO repository # GSE109145). After removal of duplicate DNA fragments and DNA fragments present in both jj and ctrl (physiological genes), 1884 unique DNA sequences were identified. Since variations in the level of histone acetylation in the promoter region positively correlate with gene transcription\textsuperscript{24,25}, we focused on peaks identified by ChIP-Seq on the promoter regions (Table 1: 255 genes). As shown in Fig. 2, the functional annotation analysis of the corresponding genes\textsuperscript{26–28} revealed an enrichment for genes involved in CNS development ($\Sigma45\%$), metabolism & homeostasis ($\Sigma31\%$), signalling ($\Sigma13\%$), response to stimuli & communication ($\Sigma5\%$), and binding ($\Sigma2\%$).

**Morphological features of the Gunn rat CII.** Since our results strongly suggested an impact of bilirubin on the genetic program of CNS maturation, we systematically followed the histological development of the cerebellum of jj rats in the attempt to interpret the genetic results. No morphological alterations between jj and ctrl were obvious at P2 (Fig. 3A,B). In both jj and ctrl animals, Purkinje cells were organized in 3–5 layers, with a round/oval shape and a reticulated cytoplasm (Fig. 3B). At P9, in spite of a conserved architecture, signs of cellular suffrage/death, microgliosis, extracellular matrix abnormalities and edema were evident in jj pups. PCs in ctrl displayed a clear definition of the plasma-membrane, cytoplasm, and nuclear areas, and a round/drop shape, and were organized in 3/1 layers. On the contrary, in jj pups, PCs were largely present in 4/2 layers, with an undefined, irregular shape. At P17, microgliosis and signs of cellular suffrage were still present in jj rats. PCs in ctrl were well differentiated, with a drop shape, and almost completely organized in a single layer, diffusely in 2/1 layers and still presenting the altered morphology described at P9 in jj. In the adult animal, the effect of CII hypoplasia was well appreciable, with a less developed structure characterized by large spaces between the folia (Fig. 3A). Microgliosis was reduced but still present. No PC’s neurites were visible in jj rats, where PCs appeared atrophic and apoptotic (Fig. 3B).

**RTqPCR analysis of selected genes.** Due to the surprising percentage of enrichment for genes involved in CNS development, we decided to confirm and quantify the epigenetic control of a selected panel of genes, by assessing their expression by RTqPCR (selected genes are those in red in Fig. 2B, in which their biological functions based on the Gene Ontology analysis are indicated. RTqPCR results are in Fig. 4). Ptk2 (protein tyrosine kinase 2 beta, considered a key gene in neurite outgrowth and elongation, synapses formation, and actin reorganization\textsuperscript{29}), was significantly down-regulated in P2 jj pups ($\Sigma2$ fold vs. age-matched ctrl, unpaired t-test with Welch correction, $p < 0.047$), normalizing thereafter. Mag (myelin-associated glycoprotein), barely detectable immediately after birth, was highly expressed in ctrl and $\Sigma2.5$ fold down-regulated in jj pups at P9 (unpaired t-test with Welch correction, $p < 0.0402$), reversing to a $\Sigma1.2$ fold up-regulation at P17 (unpaired t-test with Welch correction, $p < 0.0306$). Lami1 (intracellular adhesion molecule 1, expressed mainly by the endothelial cells forming the blood-brain barrier, involved in cell adhesion, leukocytes\textsuperscript{30} and monocytes extravasation\textsuperscript{31}, and morphogenesis) was up-regulated 1.6 fold in P17 jj rats (unpaired t-test with Welch correction, $p < 0.0416$). Similarly, we observed a $\Sigma2.2$ fold increase (unpaired t-test with Welch correction, $p < 0.0315$) of Chmp1a (charged multi-vesicular body protein 1a, regulating the neural progenitor cell proliferation\textsuperscript{32}). In adult jj CII, Col4a3 (collagenase 4a3, the major structural component of the basal membrane, involved in the extracellular matrix remodeling\textsuperscript{33}, providing the functional compartmentalization of the brain by clustering of growth factors, neurotransmitters/ions receptors, as well contributing to migration and differentiation\textsuperscript{34}), Casp6 (caspase 6 - proliferation and morphogenesis – Fig. 2B), and Arg5 (Rho GTPase-activating protein, inhibiting the cell motility and axon outgrowth via regulating the cytoskeleton dynamics\textsuperscript{35}) were upregulated $\Sigma2.5$fold (unpaired t-test with Welch correction, $p < 0.00547$), $\Sigma1.9$fold (unpaired t-test with Welch correction, $p < 0.0287$) and $\Sigma1.6$ fold (unpaired t-test with Welch correction, $p = 0.0142$) respectively. No modulation of Anxa2 (annexin2), Agrp (Agrin), and Tubb2b (Tubulin2b) was detected at any post-natal age in jj rats (data not shown). Il6 (intron region segment resulting from ChIP-Seq analysis) was also investigated. In ctrl animals the Il6 level rapidly decreases from P2 to P9, stabilizing thereafter. In jj pups, a significant down-regulation of Il6 was present immediately after birth compared to ctrl animals ($\Sigma2.9$fold, unpaired t-test with Welch correction, $p < 0.0315$), while a 1.65 fold up-regulation was noticed at P9 (unpaired t-test with Welch correction, $p < 0.0248$), normalizing later on.
| Gene Name | Gene Description | Nearest Refseq | Gene Type |
|-----------|------------------|----------------|-----------|
| Abcc10    | ATP-binding cassette, subfamily C (CFTR/MRP), member 10 | NM_001108201 | protein-coding |
| Acot13    | acyl-CoA thioesterase 13 | NM_001106111 | protein-coding |
| Acph1     | acid phosphatase 1, soluble | NM_021262 | protein-coding |
| Acph2     | acid phosphatase, testicular | NM_001107510 | protein-coding |
| Act1      | actin, alpha, cardiac muscle 1 | NM_019183 | protein-coding |
| Adra2b    | adrenoceptor alpha 2B | NM_138505 | protein-coding |
| Agrn      | agrin | NM_175754 | protein-coding |
| Abhr      | aryl-hydrocarbon receptor repressor | NM_001024285 | protein-coding |
| Aldh3a2   | aldehyde dehydrogenase 3 family, member A2 | NM_031731 | protein-coding |
| Alg11     | ALG11, alpha-1,2-mannosyltransferase | NM_001108401 | protein-coding |
| Alg8      | ALG8, alpha-1,3-glucosyltransferase | NM_001034127 | protein-coding |
| Amdhd1    | amidohydrolase domain containing 1 | NM_001191781 | protein-coding |
| Anxa2     | annexin A2 | NM_019905 | protein-coding |
| Arfgap2   | ADP-ribosylation factor GTase activating protein 2 | NM_001033707 | protein-coding |
| Arhgap4   | Rho GTase activating protein 4 | NM_144740 | protein-coding |
| Axl       | argininosuccinate lyase | NM_021577 | protein-coding |
| Atpt6e1   | ATPase, H+ transporting, lysosomal, V0 subunit e1 | NM_053578 | protein-coding |
| Atrasid   | all-trans retinoic acid-induced differentiation factor | NM_001127526 | protein-coding |
| B3galt4   | UDP-GalbetaGlcNAc beta 1,3-galactosyltransferase, polypeptide 4 | NM_133553 | protein-coding |
| Bbs2      | Bardet-Biedl syndrome 2 | NM_053618 | protein-coding |
| Bbs5      | Bardet-Biedl syndrome 5 | NM_001108583 | protein-coding |
| Bin2      | bridging integrator 2 | NM_001102223 | protein-coding |
| Bip       | biphosphoryl hydrolase-like (serine hydrolase) | NM_0011037206 | protein-coding |
| Btbd9     | bromodomain containing 9 | NM_001107453 | protein-coding |
| Cacng8    | calcium channel, voltage-dependent, gamma subunit 8 | NM_080696 | protein-coding |
| Cap1      | CAP, adenylate cyclase-associated protein 1 (yeast) | NM_022383 | protein-coding |
| Casp6     | caspase 6 | NM_031775 | protein-coding |
| Chba      | Cbl proto-oncogene C, E3 ubiquitin protein ligase | NM_001034920 | protein-coding |
| Ccmt6a    | chaperonin containing Tcp1, subunit 6 A (zeta 1) | NM_001033684 | protein-coding |
| Cdc20     | cell division cycle 20 | NM_171993 | protein-coding |
| Cer1      | ceramide synthase 1 | NM_001044230 | protein-coding |
| Chiad     | chondroadherin | NM_019164 | protein-coding |
| Chmp1a    | charged multivesicular body protein 1 A | NM_001108313 | protein-coding |
| Chrmh1    | cholinergic receptor, nicotinic, beta 1 (muscle) | NM_012528 | protein-coding |
| Ciapin1   | cytokine induced apoptosis inhibitor 1 | NM_001107689 | protein-coding |
| Cidea     | cell death-inducing DFFA-like effector a | NM_001170467 | protein-coding |
| Clpsl2    | colipase-like 2 | NM_001135002 | protein-coding |
| Cnkr1     | connector enhancer of kinase suppressor of Ras 1 | NM_001103901 | protein-coding |
| Col4a3    | collagen, type IV, alpha 3 | NM_001132579 | protein-coding |
| Col7a1    | collagen, type VII, alpha 1 | NM_001106858 | protein-coding |
| Cpm6      | copine VI (neuronal) | NM_001191113 | protein-coding |
| Cps1f3l   | cleavage and polyadenylation specific factor 3-like | NM_0011033892 | protein-coding |
| Cps1f4     | cleavage and polyadenylation specific factor 4 | NM_0011012351 | protein-coding |
| Ccrp      | CGRP receptor component | NM_053670 | protein-coding |
| Cth       | cystathionine gamma-lyase | NM_017074 | protein-coding |
| Ctr9      | CTR9 homolog, Pafl/RNA polymerase II complex component | NM_001106661 | protein-coding |
| Cyb5r1    | cytochrome b5 reductase 1 | NM_001031326 | protein-coding |
| Cyba      | cytochrome b-245, alpha polypeptide | NM_024160 | protein-coding |
| Dbl1      | damage-specific DNA binding protein 1, 127kDa | NM_171995 | protein-coding |
| Dbl2      | damage specific DNA binding protein 2 | NM_0011271346 | protein-coding |
| Ddxs      | DNA damage-induced apoptosis suppressor | NM_001126294 | protein-coding |
| Ddxs1l2   | DNA damage-inducible transcript 4 like 2 | NM_080399 | protein-coding |
| Ddx5      | DEAD (Asp-Glu-Ala-Asp) box polypeptide 55 | NM_001127132 | protein-coding |
| Ddx56     | DEAD (Asp-Glu-Ala-Asp) box helicase 56 | NM_0011004211 | protein-coding |
| Dhdds     | dehydrodolichyl diphosphate synthase subunit | NM_001101978 | protein-coding |

Continued
| Gene Name | Gene Description | Nearest Refseq | Gene Type |
|-----------|------------------|---------------|-----------|
| Dmrtc2    | DMRT-like family C2 | NM_001109140 | protein-coding |
| Dnaja1    | DnaJ (Hsp40) homolog, subfamily A, member 1 | NM_022934 | protein-coding |
| Eif3e     | eukaryotic translation initiation factor 3, subunit E | NM_001011990 | protein-coding |
| Emc3      | ER membrane protein complex subunit 3 | NM_001008355 | protein-coding |
| Emd       | emerin           | NM_012948 | protein-coding |
| Entpd6    | ectonucleoside triphosphate diphosphohydrolase 6 | NM_053498 | protein-coding |
| Eny2      | enhancer of yellow 2 homolog (Drosophila) | NM_001130580 | protein-coding |
| Ephpx2    | epoxide hydrolase 2, cytoplasmic | NM_022936 | protein-coding |
| Fam151a   | family with sequence similarity 151, member A | NM_001005558 | protein-coding |
| Fam178b   | family with sequence similarity 178, member B | NM_001122653 | protein-coding |
| Fam192a   | family with sequence similarity 192, member A | NM_001014014 | protein-coding |
| Fanca     | Fanconi anemia, complementation group A | NM_001108455 | protein-coding |
| Fbxo44    | F-box protein 44 | NM_001191576 | protein-coding |
| Fder      | ferredoxin reductase | NM_024153 | protein-coding |
| Fgfr1sp2  | FGFR1 oncogene partner 2 | NM_201421 | protein-coding |
| Fkhbp6    | FK506 binding protein 6 | NM_001105922 | protein-coding |
| Fxom1     | forkhead box M1 | NM_051633 | protein-coding |
| Fyoo1     | FYVE and coiled-coil domain containing 1 | NM_001106870 | protein-coding |
| Gamt      | guanidinosuccinate N-methyltransferase | NM_012793 | protein-coding |
| Gdf1      | growth differentiation factor 1 | NM_001044240 | protein-coding |
| Gja4      | gap junction protein, alpha 4 | NM_021654 | protein-coding |
| Gja4      | gap junction protein, delta 4 | NM_001100487 | protein-coding |
| Gna15     | guanine nucleotide binding protein, alpha 15 | NM_053542 | protein-coding |
| Gng5      | guanine nucleotide binding protein (G protein), gamma 5 | NM_024377 | protein-coding |
| Gmnt      | glycine N-methyltransferase | NM_017084 | protein-coding |
| Gnpat     | glycereinophosphate O-acyltransferase | NM_053410 | protein-coding |
| Gso2      | golgi SNAP receptor complex member 2 | NM_051685 | protein-coding |
| Gpalpp1   | GPALPP motifs containing 1 | NM_001024875 | protein-coding |
| Gtj2c1    | general transcription factor IIE, polypeptide 1, alpha | NM_001100556 | protein-coding |
| Gtfs1     | gametocyte specific factor 1 | NM_001079707 | protein-coding |
| Gzf1      | GDNF-inducible zinc finger protein 1 | NM_001017788 | protein-coding |
| Hefc1r1   | host cell factor C1 regulator 1 (KPO1-dependent) | NM_001104942 | protein-coding |
| Hgel2a    | HGI1 homolog a, inducible domain family, member 2A | NM_001106102 | protein-coding |
| Hist3h2a  | histone cluster H2a | NM_021840 | protein-coding |
| Hist3h2bb | histone cluster 3, H2bb | NM_001109641 | protein-coding |
| Hoxc8     | homeobox C8 | NM_001177326 | protein-coding |
| Hoodi0    | homeo box D10 | NM_001107094 | protein-coding |
| Hrg       | histidine-rich glycoprotein | NM_133428 | protein-coding |
| Icam1     | intercellular adhesion molecule 1 | NM_012967 | protein-coding |
| Idua      | iduronidase, alpha-L- | NM_001172084 | protein-coding |
| Ift122    | intratubular transport 122 | NM_001099456 | protein-coding |
| Ikzf5     | Ikaros family zinc finger 5 | NM_001107555 | protein-coding |
| Il17rb    | interleukin 17 receptor B | NM_001107290 | protein-coding |
| Ig6a      | integrin, alpha 4 | NM_001107737 | protein-coding |
| Iggr1     | jagunal homolog 1 | NM_001044272 | protein-coding |
| Itb       | jumping translation breakpoint | NM_019213 | protein-coding |
| Kb15      | type II keratin K15 | NM_001008825 | protein-coding |
| Kcone5    | potassium channel, voltage-gated Iak-related subfamily E regulatory beta subunit 5 | NM_001101003 | protein-coding |
| Kdelr1    | KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 1 | NM_001017385 | protein-coding |
| Ksao0895l | hypothetical protein LOC688736 | NM_001044292 | protein-coding |
| Kfj11     | kinesin family member 11 | NM_001169112 | protein-coding |
| Kfj18b    | kinesin family member 18B | NM_001039019 | protein-coding |
| Kldel1    | killer cell lectin-like receptor, subfamily D, member 1 | NM_012745 | protein-coding |
| Krt33b    | keratin 33B | NM_00108819 | protein-coding |
| Lars2     | leucyl-tRNA synthetase 2, mitochondrial | NM_001108877 | protein-coding |

Continued
| Gene Name | Gene Description | Nearest Refseq   | Gene Type       |
|-----------|------------------|-----------------|-----------------|
| Leng1     | leukocyte receptor cluster (LRC) member 1 | NM_001106218    | protein-coding  |
| Lhx1      | LIM homeobox 1    | NM_145880       | protein-coding  |
| LOC100912214 | uncharacterized LOC100912214 | NR_131101 | protein-coding  |
| LOC103689982 | lysophospholipid acyltransferase 7 | NM_001313940 | protein-coding  |
| LOC288913 | similar to LEYDIG CELL TUMOR 10 KD PROTEIN | NM_198728 | protein-coding  |
| LOC498154 | hypothetical protein LOC498154 | NM_001025033 | protein-coding  |
| LOC688925 | similar to Glutathione S-transferase alpha-4 | NM_001270386 | protein-coding  |
| Lrc14     | leucine rich repeat containing 14 | NM_001024554    | protein-coding  |
| Lrc27     | leucine rich repeat containing 27 | NM_001113789    | protein-coding  |
| Lrc36     | leucine rich repeat containing 36 | NM_001004088    | protein-coding  |
| Lrc51     | leucine rich repeat containing 51 | NM_001106284    | protein-coding  |
| Ly6d3     | Ly6/Plasmin domain containing 3 | NM_021759       | protein-coding  |
| Lziel     | leucine zipper and CTNNB1I domain containing | NM_001013241 | protein-coding  |
| Lziif1    | leucine zipper transcription factor-like 1 | NM_001024266 | protein-coding  |
| Lhox1     | LIM homeobox 1    | NM_145880       | protein-coding  |
| LOC100912214 | uncharacterized LOC100912214 | NR_131101 | protein-coding  |
| LOC103689982 | lysophospholipid acyltransferase 7 | NM_001313940 | protein-coding  |
| LOC288913 | similar to LEYDIG CELL TUMOR 10 KD PROTEIN | NM_198728 | protein-coding  |
| LOC498154 | hypothetical protein LOC498154 | NM_001025033 | protein-coding  |
| LOC688925 | similar to Glutathione S-transferase alpha-4 | NM_001270386 | protein-coding  |
| Lrc14     | leucine rich repeat containing 14 | NM_001024554    | protein-coding  |
| Lrc27     | leucine rich repeat containing 27 | NM_001113789    | protein-coding  |
| Lrc36     | leucine rich repeat containing 36 | NM_001004088    | protein-coding  |
| Lrc51     | leucine rich repeat containing 51 | NM_001106284    | protein-coding  |
| Ly6d3     | Ly6/Plasmin domain containing 3 | NM_021759       | protein-coding  |
| Lziel     | leucine zipper and CTNNB1I domain containing | NM_001013241 | protein-coding  |
| Lziif1    | leucine zipper transcription factor-like 1 | NM_001024266 | protein-coding  |
| Maf1      | MAF1 homolog, negative regulator of RNA polymerase III | NM_001014085 | protein-coding  |
| Mag       | myelin-associated glycoprotein | NM_017190       | protein-coding  |
| Mal       | mal, T-cell differentiation protein | NM_012798       | protein-coding  |
| Mboat7    | membrane bound O-acyltransferase domain containing 7 | NM_0013147978 | protein-coding  |
| Memp1     | mast cell-expressed membrane protein 1 | NM_001134602 | protein-coding  |
| Mus1      | muscle-enriched antigen 1 | NM_001044286 | protein-coding  |
| Mef11     | mediator complex subunit 11 | NM_00105799 | protein-coding  |
| Mir137    | microRNA 137 | NR_031883 | ncRNA |
| Mir207    | microRNA 207 | NR_032107 | ncRNA |
| Mir338    | microRNA 338 | NR_031783 | ncRNA |
| Mir3562   | microRNA 3562 | NR_03744 | ncRNA |
| Misip     | mitotic spindle positioning | NM_001109284 | protein-coding  |
| Mrpl43    | mitochondrial ribosomal protein L43 | NM_001107598 | protein-coding  |
| Mrps18b   | mitochondrial ribosomal protein S18B | NM_212534 | protein-coding  |
| Mrps25    | mitochondrial ribosomal protein S25 | NM_001025408 | protein-coding  |
| Mt2A      | metallothionein 2A | NM_001137564 | protein-coding  |
| Mt5       | metallothionein 3 | NM_053968 | protein-coding  |
| Mtpr3     | mitochondrial transcription termination factor 3 | NM_199387 | protein-coding  |
| Mtfl      | metal-regulatory transcription factor 1 | NM_001088677 | protein-coding  |
| Mtfl2     | metal response element binding transcription factor 2 | NM_001108998 | protein-coding  |
| Myeon2    | myeloma overexpressed 2 | NM_001109044 | protein-coding  |
| Naa38     | N(alpha)-acyltransferase 38, NatC auxiliary subunit | NM_001105794 | protein-coding  |
| Ncd1     | nuclear cap binding protein subunit 1 | NM_001101475 | protein-coding  |
| Ncoa4     | nuclear coactivator 4 | NM_001034007 | protein-coding  |
| Ndr1      | NADPH dependent di flavin oxidoreductase 1 | NM_001107818 | protein-coding  |
| Nafl8     | NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8 | NM_001106360 | protein-coding  |
| Naflf5    | NADH dehydrogenase (ubiquinone) Fe-S protein 5 | NM_001030052 | protein-coding  |
| Naflf3    | NADH dehydrogenase (ubiquinone) flavoprotein 3 | NM_022607 | protein-coding  |
| Nipsnap1  | nipsnap homolog 1 (C. elegans) | NM_001100730 | protein-coding  |
| Nme3      | NME/NM23 nucleoside diphosphate kinase 3 | NM_053507 | protein-coding  |
| Nmi       | N-myc (and STAT) interactor | NM_001034148 | protein-coding  |
| Nmu       | neuromedin U | NM_022239 | protein-coding  |
| Nocr1     | NIH1/RPN12 binding protein 1 homolog | NM_199086 | protein-coding  |
| Nocl1     | nucleolar and coiled-body phosphoprotein 1 | NM_022869 | protein-coding  |
| N2c2ap    | nuclear receptor 2C2-associated protein | NM_001047104 | protein-coding  |
| Nsl1      | NSL1, MIS12 kinetochore complex component | NM_001109083 | protein-coding  |
| Ntpcr     | nucleoside-triphosphatase, cancer-related | NM_001134573 | protein-coding  |
| Ntr1      | neurotensin receptor 1 | NM_001108967 | protein-coding  |
| Nubp2     | nucleotide binding protein 2 | NM_0011011891 | protein-coding  |
| Nufl2     | nifedix (nucleoside diphosphate linked moiety X)-type motif 2 | NM_207956 | protein-coding  |
| Ohfr437   | olfactory receptor 437 | NM_001109347 | protein-coding  |
| Ohfr760   | olfactory receptor 760 | NM_001001069 | protein-coding  |
| Gene Name | Gene Description | Nearest Refseq | Gene Type |
|-----------|------------------|----------------|-----------|
| Ovca2     | ovarian tumor suppressor candidate 2 | NM_001109036 | protein-coding |
| Pdalna3   | protocadherin alpha 3 | NM_053941 | protein-coding |
| Pctp      | phosphatidylcholine transfer protein | NM_017225 | protein-coding |
| Pex1      | peroxisomal biogenesis factor 1 | NM_001109220 | protein-coding |
| Phlda2    | pleckstrin homology-like domain, family A, member 2 | NM_001100521 | protein-coding |
| Phldb3    | pleckstrin homology-like domain, family B, member 3 | NM_001191622 | protein-coding |
| Pigg      | phosphatidylinositol glycan anchor biosynthesis, class P | NM_001099758 | protein-coding |
| Plcsc2    | phosphatidylinositol-specific phospholipase C, X domain containing 2 | NM_001134481 | protein-coding |
| Plp2      | pro tease lipolipid protein 2 (colonie epithelium-enriched) | NM_207601 | protein-coding |
| Pmgf      | polyamine-modulated factor 1 | NM_001191568 | protein-coding |
| Pnldc1    | poly(A)-specific ribonuclease (PARN)-like domain containing 1 | NM_001025724 | protein-coding |
| Pob3d     | polymerase (RNA) III (DNA directed) polypeptide D | NM_001031653 | protein-coding |
| Pou6f1    | POU class 6 homeobox 1 | NM_001105746 | protein-coding |
| Ppp1v11   | protein phosphatase 1, regulatory (inhibitor) subunit 11 | NM_212542 | protein-coding |
| Ppt2      | palmityl-protein thi oesterase 2 | NM_019367 | protein-coding |
| Pmng4     | proteasome (prosome, macropain) assembly chaperone 4 | NM_001109543 | protein-coding |
| Ptc01     | pentatricopeptide repeat domain 1 | NM_001109665 | protein-coding |
| Ptk2b     | protein tyrosine kinase 2 beta | NM_017318 | protein-coding |
| Qk        | quaking | NM_001115021 | protein-coding |
| Rab3gap2  | RAB3 GTPase activating protein subunit 2 | NM_00108294 | protein-coding |
| Rab5c     | RAB5C, member RAS oncogene family | NM_00105880 | protein-coding |
| Rad51ap1  | RAD51 associated protein 1 | NM_001079711 | protein-coding |
| Rankp10   | RAN binding protein 10 | NM_001135875 | protein-coding |
| Rec8      | REC8 meiotic recombination protein | NM_00101916 | protein-coding |
| Rbl2      | replication factor C (activator 1) 2 | NM_053786 | protein-coding |
| G3437443  | similar to mKIAA0319 protein | NM_001197023 | protein-coding |
| G3439188  | similar to hypothetical protein RC001833 | NM_001108129 | protein-coding |
| G3439676  | similar to RIKEN cDNA 5730469M10 | NM_001014140 | protein-coding |
| G34311703 | similar to sid2057p | NM_001013898 | protein-coding |
| G34359334 | similar to hypothetical protein FLJ20519 | NM_001007638 | protein-coding |
| G343559909 | G343559909 | NM_00108678 | protein-coding |
| G34360608 | similar to novel protein | NM_001109280 | protein-coding |
| G3436283  | G3436283 | NM_001108314 | protein-coding |
| G34363714 | G34363714 | NM_001126297 | protein-coding |
| G34364036 | similar to RIKEN cDNA 3010026C09 | NM_001109030 | protein-coding |
| Rbc2      | RIB43 A domain with coiled-coils 2 | NM_001013949 | protein-coding |
| Rnf40     | ring finger protein 40, E3 ubiquitin protein ligase | NM_153471 | protein-coding |
| Rph3a     | rabphilin 3A | NM_133518 | protein-coding |
| Rpi27     | ribosomal protein L27 | NM_022514 | protein-coding |
| Rpl27a    | ribosomal protein L27a | NM_001106290 | protein-coding |
| Rpry1     | ring finger and SPRY domain containing 1 | NM_001100945 | protein-coding |
| Raps3     | relaxin/insulin-like family peptide receptor 3 | NM_00108310 | protein-coding |
| Sart3     | squamous cell carcinoma antigen recognized by T-cells 3 | NM_001107156 | protein-coding |
| Scyl      | selenocysteine lyase | NM_001007755 | protein-coding |
| Sert1     | Sertoli cell protein 1 | NR_130708 | ncRNA |
| Sfot73    | sideroflexin 3 | NM_022948 | protein-coding |
| Skn2      | srk kinase associated phosphoprotein 2 | NM_130413 | protein-coding |
| Slc19a2   | solute carrier family 19 (thiamine transporter), member 2 | NM_001030024 | protein-coding |
| Slc25a54  | solute carrier family 25, member 54 | NM_001109640 | protein-coding |
| Slc43a3   | solute carrier family 43, member 3 | NM_001107743 | protein-coding |
| Slc5a6    | solute carrier family 5 (sodium/multivitamin and iodide cotransporter), member 6 | NM_130746 | protein-coding |
| Slc6a20   | solute carrier family 6 (proline IMINO transporter), member 20 | NM_133296 | protein-coding |
| Slc6a3    | solute carrier family 6 (neurotransmitter transporter), member 3 | NM_012694 | protein-coding |
| Snrnp35   | small nuclear ribonucleoprotein 35 (U11/U12) | NM_001014127 | protein-coding |
| Snrpb2    | small nuclear ribonucleoprotein polypeptide B² | NM_001108592 | protein-coding |

Continued
in vitro hyperbilirubinaemia on CNS development has been only marginally envisaged, and evaluated mostly by
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Discussion

Cll hypoplasia is a hallmark of hyperbilirubinemia in rodents26–29, and cerebellar involvement with morphological
and behavioral abnormalities has also been reported in severely hyperbilirubinemic neonates30–32. Inflammation
and oxidative stress are considered the major mechanisms of bilirubin neurotoxicity, whereas the impact of
hyperbilirubinemia on CNS development has been only marginally envisaged, and evaluated mostly by in vitro
experiments33,34.

Unexpectedly, the known inflammatory or oxidant effectors of bilirubin neurotoxicity have been not identified
in our data (ChIP-Seq, followed by Gene Ontology analysis), revealing that 45% of genes displaying a Histone
acetylation are related to CNS development. Indeed, only 3 genes among all the 255 identified TSS-

| Gene Name    | Gene Description               | Nearest Refseq     | Gene Type      |
|--------------|--------------------------------|--------------------|----------------|
| Spag7        | sperm associated antigen 7      | NM_001107016       | protein-coding |
| Spata33      | spermato genesis associated 33 | NM_001106195       | protein-coding |
| Spata5       | spermato genesis associated 5  | NM_001108549       | protein-coding |
| SpiC         | Spi-C transcription factor     | NM_001108080       | protein-coding |
| Stam         | signal transducing adaptor     | NM_001109121       | protein-coding |
| Stk19        | serine/threonine kinase 19     | NM_001013197       | protein-coding |
| Susd3        | sushi domain containing 3      | NM_001107341       | protein-coding |
| Tada3        | transcriptional adaptor 3      | NM_001025734       | protein-coding |
| Taftl        | TAF6-like RNA polymerase II, p300/CBP-associated factor | NM_001107575 | protein-coding |
| Taxislip3    | TaxI (human T-cell leukemia virus type I) binding protein 3 | NM_001025419 | protein-coding |
| Tbc1d25      | TBC1 domain family, member 25 | NM_001106955       | protein-coding |
| Tbc2         | tubulin folding cofactor B     | NM_001040180       | protein-coding |
| Tem4         | thioredoxin superfamily member | NM_001025017       | protein-coding |
| Tmem109      | transmembrane protein 109      | NM_001007736       | protein-coding |
| Tmem126a     | transmembrane protein 126 A    | NM_001011557       | protein-coding |
| Trxax-ps1    | tenascin XA, pseudogene 1      | NR_024118          | pseudo         |
| Trappc1      | trafficking protein particle complex 1 | NM_001039378 | protein-coding |
| Trim23       | tripartite motif-containing 23 | NM_001106037       | protein-coding |
| Trip13       | thyroid hormone receptor interactor 13 | NM_001011930 | protein-coding |
| Trip4        | thyroid hormone receptor interactor 4 | NM_001134981 | protein-coding |
| Trmt112      | tRNA methyltransferase 11-2 homolog (S. cerevisiae) | NM_001106330 | protein-coding |
| Tsc2         | tuberous sclerosis 2           | NM_012680          | protein-coding |
| Tsgd2        | thiosulfate sulfurtransferase (rhodanese)-like domain containing 2 | NM_001108663 | protein-coding |
| Ttc3         | tetratricopeptide repeat domain 3 | NM_001108315 | protein-coding |
| Tubal3a      | tubulin, alpha 3A              | NM_001040008       | protein-coding |
| Tubal4a      | tubulin, alpha 4A              | NM_001007004       | protein-coding |
| Tub82b       | tubulin, beta 2B class IIb     | NM_001013886       | protein-coding |
| Ufip2        | UFM1-specific peptidase 2      | NM_001014142       | protein-coding |
| Vmp1         | vacuole membrane protein 1     | NM_138839          | protein-coding |
| Yvra7        | von Willebrand factor A domain containing 7 | NM_212499 | protein-coding |
| Zbcb26       | zinc finger and BTB domain containing 26 | NM_001107840 | protein-coding |
| Zfp597       | zinc finger protein 597        | NM_153732          | protein-coding |
| Zscan21      | zinc finger and SCAN domain containing 21 | NM_001012021 | protein-coding |

Table 1. Full list of ChIP-Seq TSS-Promoter genes.

The down-regulation of Mag has been reported in in vitro studies, in agreement with the defective myelination
observed both in bilirubin neurotoxicity models33,34, and in neonates35. Mag down-regulation is also a known
consequence of bilirubin-induced perturbation of the oligodendrocytes maturation. A possible additional link
between what has been previously described and the present results is the fact that histone acetylation is a known
mechanism controlling oligodendrocyte differentiation and myelin production, both in physiological CNS develop-
ment and in repair processes after demyelination6,36.

Our data are in agreement with the literature also in relation to Il6, whose intron sequence was identified by
ChIP-Seq analysis. Il6 is a well-known effector of bilirubin neurotoxicity and possibly linked with the reported
defective myelination. In fact, apart from the possible inflammatory activity, Il6 is involved in oligodendroge-
nesis37,38, a process active up to P45 in rodents and 2 years in humans39, and reactivated in pathologiacal conditions.
During reactivation, injured neurons and oligodendrocytes may reactivate myelin synthesis by overexpressing Il6
and its receptor (Il6r/CD126), restoring normal behavior in injured animals40,41.
Both Mag and Il6 present a fluctuating behavior, being significantly down-regulated in the early post-natal life, and reverting thereafter to the level of age-matched controls (Fig. 4). Notably, in our work, IL6 modulation (P9) precedes Mag increase (P17), supporting the inductor role of Il6 in myelination described in the literature 10,40.

The fluctuating expression of Il6 and Mag (firstly up-, then down regulated), is present also for H3K14Ac levels, increasing at P9, and reverting under the level of age-matched controls at P17, and normalizing in the adult age.

The regulation of the other genes is more difficult to be analyzed since they are very new in the bilirubin field and no data are provided by literature. While we still have to confirm the role of the various genes identified in this study through methods such as gene silencing in vitro, our work suggests that the epigenetic impairment of neurodevelopmental processes in hyperbilirubinemia may be a relevant mechanism of bilirubin neurotoxicity. It is worth mentioning that Chmp1a, Arghap4, Casp6, Ptk2, Col4a3 are genes involved in key steps of brain development as proliferation, migration, morphogenesis, neurite outgrowth and elongation, synaptogenesis, extracellular matrix formation and compartmentalization, as well the pathological axonal degeneration and apoptosis observed in jj rats. By adding epigenetic dysregulation to the list of the mechanisms related to bilirubin-induced neuronal damage, we can confirm and expand the concept of a widespread toxic effect of the pigment on the CNS43, improving our understanding of the cellular and molecular mechanisms of bilirubin induced damage to CNS.

Materials and Methods

Animals. Gunn rats (Hds Blue:Gunn-UDPGT, P2, 9, 17; P ± 1 day. Adult = more than 1 year old) were obtained from the SPF animal facility of CBM S.c.a.r.l. (AREA Science Park, Basovizza). Ages were selected based on previous evidence26,44. Animals were housed in a temperature-controlled environment (22 ± 2°C), on a 12 hours light/dark schedule, and ad-libitum access to food and water. The study was approved by the animal care and use committee of the CBM Scarl and the competent Italian Ministry. All procedures were performed according to the Italian Law (decrees 87-848) and European Community directive (86-606-ECC). Maximal effort to minimize the number of the animals used and their sufferance was done.
TSB, cBf and Cerebellum weight quantification. Serum and Cll were collected as previously described \(^{26,46}\). In brief, blood samples were collected during the sacrifice (decapitation under urethane anaesthesia 1.0–1.2 g/kg IP) and centrifuged at 2000 rpm, 20 min RT. Total serum bilirubin (TSB) was quantified by the diazo reaction, as previously described \(^{26}\). Free bilirubin was calculated (cBf) by applying the formula and the albumin-bilirubin dissociation constants for Gunn pups detailed in literature \(^{14}\). Cerebellum was dissected immediately after the sacrifice, and the weight recorded by a precision balance.

Western blot analysis of the levels of H3K14Ac. Western blot was performed as previously described \(^{44,45}\). In brief, Cll were mechanically homogenized by glass–glass Dounce (in 0.25 M sucrose, 40.2 mM KH\(_2\)PO\(_4\), 9.8 mM KHPO\(_4\), 1 mM EDTA, 0.1 mM DTT, pH 7.4), and total protein concentration quantified by the Bicinchoninic Acid Protein Assay following the supplier instruction (B-9643 and C2284, Sigma, Missouri, USA). 25μg of Cll whole extract proteins were denatured (10% of 3-mercaptoethanol -Sigma Chemical, St. Louis, MO, USA, plus 5 min boiling), separated by 12% SDS-PAGE by electrophoresis in a Hoefer SE 250 System (Amersham...
BioSciences, UK), and electro-transferred onto immune-blot PVDF membranes (0.2 μm; Whatman Schkleicher and Schuell, Dassel, Germany) at 100 V for 60 min (Bio-Rad Laboratories, Hercules, CA, USA). Efficiency of the transfer was assessed by lack of Coomassie blue coloration of the gel after blotting, and Ponceau staining of the PVDF membrane (both chemicals: Sigma, St. Louis, MO, USA). After blocking (1.5 hrs, RT in blocking solution: 3% defatted milk in 0.2% Tween 20; 20 mM Tris-HCl pH 7.5; 500 mM NaCl), membranes were incubated O/N at 4 °C with the polyclonal anti-acetyl histone H3 (lys14) antibody (07-353, Merck Millipore, Temecula, CA, USA; final concentration 0.7 μg/mL). The day after, membranes were washed 3×5 min in blocking buffer, then incubated 2hrs with the secondary antibody anti-rabbit IgG peroxidase (Dako, Agilent Technologies, Santa Clara, CA, USA, final concentration 0.0625 μg/mL) in blocking solution. The signal was revealed by chemiluminescence (ECL-Plus Western blotting Detection Reagents, GE-Healthcare Bio-Science, Italy) and visualized on X-ray films (BioMax Light, Kodak Rochester, NY, USA). The results were normalized vs. the actin signal, visualized incubating the same membrane used for revealing the H3K14Ac with the anti-actin antibody A2066 (sigma- Chemical, St. Louis, MO, USA; final concentration 0.07 μg/mL, MW 42KDa). Bands intensity was quantified by the Scion Image software (GE Healthcare Europe GmbH, France).

ChIP-Seq analysis. The 07-353 anti-H3K13Ac antibody used for Western blot analysis was also used to perform chromatin immunoprecipitation, followed by DNA sequencing (ChIP-Seq – full result available on GEO repository # GSE109145). Chromatin immunoprecipitation (ChIP) was performed following the Magna ChiP™ G Tissue Kit (#17-20000, Merck Millipore, Temecula, CA, USA) procedure and applying the same Ab used in Western blot. Ctl tissue (60 mg) was homogenized, DNA sheared (average size of 100–400 bp, by Sonopuls HD 3100, Bandelin, Germany, sonicator. Power 50%, 15″ × 18 cycles, 10″ pause between each cycle, on ice), cross-linked with 1% formaldehyde (5′, RT), and protein–DNA complexes immune-precipitated (5 μL, 07-353 Ab, Merck Millipore, Temecula, CA, USA) by G magnetic beads on the magnetic rack (LSKMAGS08 Pure Proteome™ Magnetic Stand, Merck Millipore, Temecula, CA, USA). Protein-DNA crosslink was reversed (proteinase K, 62 °C, 2 h; plus 95°C × 10′), and DNA stored at –20°C until use. As suggested by the manufacturer, the efficiency and specificity of the ChIP procedure were assessed by Western blot, and Real Time PCR (RTqPCR). Samples were quantified by Quant-iTTM PicoGreen® dsDNA Kits (Thermo Fisher Scientific, Waltham, MA, USA), according to manufacturer’s instruction.

Libraries were prepared by using the NEBNext® UltraTM II DNA Library Prep Kit from Illumina® (E7645, New England BioLabs®, Inc, MA, USA), following the manufacturer’s instructions starting from 10 ng of fragmented DNA. After end repair and adapter ligation, adapter-ligated DNA clean-up (without size-selection, Agencourt AMPure XP magnetic beads, Beckman Coulter Life Sciences, CA, USA), library enrichment (98°C × 30 sec; 98°C × 10 sec plus 65°C × 75 min × 10 cycles; 65°C × 5 min, in a Bio-Rad thermal cycler, Bio-Rad, Richmond, CA, USA), and PCR clean up (Agencourt AMPure XP magnetic beads, Beckman Coulter Life Sciences,
equation, taking into account the efficiencies of the individual genes 49,50. The results were normalized to the iQ Software, version 3.1 (Bio-Rad Laboratories, Hercules, CA, USA) by the Pfaffl modification of the ΔΔ curve analysis was performed to assess product specificity. The relative quantification was made using the iCycler ethanol 2 Hercule, CA, USA) in presence of 25 ng of cDNA, sense and antisense gene-specific primers (250 nM each), in available in GenBank (Table 2). RtqPCR was performed in an iCycler iQ thermocycler (Bio-Rad Laboratories, using the Beacon designer 8.1 software (Premier Biosoft International, Palo Alto, CA, USA) on rat sequences instruction in a thermal cycler (Gene Amp PCR System 2400, Perkin-Elmer, Boston, MA, USA) at 25 °C for 2 min; 70% ethanol 1 min; 100% ethanol 2 min; 95% ethanol 2 × 2 min; 80% ethanol 1 × 2 min; 70% ethanol 1 × 2 min; H2O 2 × 5 min) by incubating the slices for 1 hr in cresyl violet solution (0.1% cresyl violet powder, 10 drops glacial acetic acid in H2O). After washing (twice H2O), differentiation (75% ethanol, 95% ethanol plus 5% chloroform, 3 drops glacial acetic acid) and dehydration (100% ethanol 2 × 5 min; xylol 2 × 5 min), slices were mounted (Eukitt 03989, SIGMA Aldrich). Pictures were collected by a D-Sight plus image digital microscope & scanner (Menarini Diagnostics, Firenze, Italy). Histology was read by 3 independent pathologists, blinded to experimental design.

**Histology and morphometric analysis.** Immediately after animals sacrificed, the brain was removed from the skull and fixed in 4% formalin buffered solution (4% formaldehyde 37%, 33 nM NaH2PO4, 46 mM Na2HPO4), then embedded in paraffin. Sagittal sections of the brain (3–5 μm) were obtained by a microtome (Microm-hm 340e-BioOptica, Milan, Italy), affixed on the glass slides and dried at 60 °C for 1 hour. Hematoxylin and eosin stain (H&E) was performed by a Leica ST5020 Multistainer (Leica Microsystem, Milan, Italy). Cresyl violet (Nissl) stainning was performed manually on hydrated sections (xylol 3 × 5 min; 100% ethanol 2 × 2 min; 95% ethanol 2 × 2 min; 80% ethanol 1 × 2 min; 70% ethanol 1 × 2 min; H2O 2 × 5 min) by incubating the slices for 1 hr in cresyl violet solution (0.1% cresyl violet powder, 10 drops glacial acetic acid in H2O(MQ). After washing (twice H2O), differentiation (75% ethanol, 95% ethanol plus 5% chloroform, 3 drops glacial acetic acid) and dehydration (100% ethanol 2 × 5 min; xylol 2 × 5 min), slices were mounted (Eukitt 03989, SIGMA Aldrich). Pictures were collected by a D-Sight plus image digital microscope & scanner (Menarini Diagnostics, Firenze, Italy). Histology was read by 3 independent pathologists, blinded to experimental design.

**RtqPCR on selected genes.** RtqPCR was performed as previously described26,43. Total RNA extraction (Eurogold RNA Pure reagent, Euroclone, Milan, Italy) and retro-transcription (1 μg RNA, High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Monza, Italy) were performed following the manufacturer instruction in a thermal cycler (Gene Amp PCR System 2400, Perkin-Elmer, Boston, USA) at 25 °C for 5 min, 37 °C for 120 min, and 85 °C for 5 min. The final cDNA was stored at 20 °C until use. Primers were designed using the Beacon designer 8.1 software (Premier Biosoft International, Palo Alto, CA, USA) on rat sequences available in GenBank (Table 2). RtqPCR was performed in an iCycler iQ thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) in presence of 25 ng of cDNA, sense and antisense gene-specific primers (250 nM each), in SSOAdvance SYBER green supermix (Bio-Rad Laboratories, Hercules, CA, USA). Amplification protocol was 95 °C × 3 min, 40 cycle of 95 °C × 20 sec; 60 °C × 20 sec and 72 °C × 30 sec, followed by 72 °C × 5 min. Melting curve analysis was performed to assess product specificity. The relative quantification was made using the iCycler iQ Software, version 3.1 (Bio-Rad Laboratories, Hercules, CA, USA) by the Pfaffl modification of the ΔΔCT equation, taking into account the efficiencies of the individual genes 49,50. The results were normalized to the housekeeping genes and the levels of mRNA were expressed relative to a reference sample50,51.

**Statistics.** The statistical analysis was performed by GraphPad InStat for Windows (GraphPad Software, Inc, La Jolla, CA, USA). The ANOVA test, followed by Tukey-Kramer multiple comparison tests, was used to analyse TSB, cBF, and Cll weight during the development. The unpaired two-tailed Student’s t-test, based on unequal variance, was applied to evaluate the difference between jj and controls at the same age (Western blot, RtqPCR). All data are expressed as mean ± S.D. of multiple biological repetition. A p-value lower than 0.05 was considered statistically significant.

**Data Availability**

ChIP-Seq – full result available on GEO repository # GSE109145.

| Gene | Accession number | Forward | Revers | Efficiency | Amplicon length (bp) |
|------|------------------|---------|--------|------------|---------------------|
| Agrn | NM_175754        | TACCCTGCACTCTGATT | TTCTCATCCAATAACACATT | 98.5 | 87 |
| Arhgap4 | NM_144740 | CTTGGAGGACATCATCATATC | GTTGAGAAGGTGGAAGAG | 88 | 75 |
| Anxa2 | NM_019965        | CTACGTCCAGAAATCCTG | AAGTTGTTGTAAGGTTTGC | 99.8 | 94 |
| Casp6 | NM_031775        | CACATGAGGCCTCTACAGA | AGTTCTCCCTCCTTGTG | 102.2 | 78 |
| Clmp1a | NM_001083313 | ATCACTTACAGGTTAGG | TACCTTGACAAACATCTGTA | 98.2 | 122 |
| Coda3 | NM_00135759      | TCACCCACTGCAATCTCTTA | CGACAGCCATGTAAAGGTA | 94.5 | 83 |
| Icam1 | NM_012967        | ACCTACTACATACCTTAC | ATGAGACTCCATGTTGGA | 96.3 | 91 |
| Mag | NM_017190       | ACATCCACACTTCTGTATC | CTGATTCCGTCAGGAATG | 96.2 | 90 |
| Ptk2b | NM_017318        | TGTCCTACAGAACATAAA | GAACCTTCTCTCTTGTG | 93.1 | 88 |
| Tub2b | NM_001013886     | CAGTTGGAAGAAGGAAGA | AGTTGTTACATTTGATGATTACG | 107.5 | 111 |
| Il6 | NM_012589.1     | GCCACACCAAGAAGGAAGATC | TCCCTGTGAAATGCTGCC | 107.7 | 161 |
| Hprt | NM_012583.2      | AGACTGAAGACTGCTGTGAATGAC | GGCTGTACTGCTGACCAAG | 94.9 | 163 |

Table 2. Primers specification.
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**Acknowledgements**

SG was supported in part by an internal grant from the Italian Liver Foundation. EV was supported in part by an internal grant from the Italian Liver Foundation, in part by the Università degli Studi di Trieste. We thanks the Alessandra Bramante and Andrea Lorenzon from the local SPF animal facility of CBM S.c.a.r.l. (AREA Science Park, Basovizza) for their support with the animal procedures, Dr. Sean M. Riordan (Mercy Children Hospital, Kansas City, MO, USA), for the final revision of the Ms. and the editing of the English, and Dr. Paola Ostano (Fondazione Edo ed Elvo Tempia Valenta, Biella) for the informatics support in loading the data on GEO.

**Author Contributions**

E.V. designed research, performed research, analyzed data. S.Z. performed research. T.M. performed research. F.T. analyzed data. C.B. performed research. A.D. Contributed new reagents/analytic tools. F.Z. performed research, analyzed data. C.T. wrote the paper. S.G. designed research, performed research, analyzed data, and wrote the paper. All authors read and approved the final version of the manuscript.

**Additional Information**

**Competing Interests:** The authors declare no competing interests.

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