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by the epigenetic configuration of target patterning genes, allowing long-term maintenance of intrinsic positional values in spite of continuously changing extrinsic signals.

1. Introduction

A remarkable feature of vertebrate neural development is the early subdivision of the neuroectoderm along its rostrocaudal and dorsoventral axes into an orderly array of sharply delimited domains expressing region-specific combinations of transcription factor-encoding genes [1,2]. For example, expression of *Otx1/2* genes identifies presumptive anterior (forebrain and midbrain) territories [3], whereas *Hox* genes are expressed within posterior (hindbrain and spinal cord) neuroectoderm [4,5]. Other gene families define positional identities along the neural tube dorsoventral axis [2]. Eventually, various combinations of transcription factors become expressed in different areas of the neural tube, leading to specification of cellular compartments with distinct transcriptional programmes and developmental fates [6].

Transcription factors involved in the specification of positional identities acquire their spatially limited expression domains in response to the action of a number of diffusible morphogens that are distributed with concentration gradients across the neural tissue [2,7,8]. Among them, retinoic acid (RA) is a key morphogen controlling rostrocaudal neural patterning [7,9,10]. RA is a product of vitamin A metabolism that is present with a posterior-high to anterior-low gradient in early vertebrate embryos due to the presence of RA-synthesizing enzymes (such as Aldh1a2) in caudal regions and RA-degrading enzymes (such as Cyp26a1) in anterior regions [9–11]. The posterior neural tube develops abnormally in embryos with reduced levels of RA [12–15]. Furthermore, embryos exposed to exogenous RA during early developmental stages undergo dramatic losses of forebrain structures and expansion of posterior neural structures [16–18]. RA regulates gene transcription by binding retinoic acid receptors (RARs), which interact with retinoic acid response elements (RAREs) within regulatory regions of RA-responsive genes to control their expression [11]. In particular, well-characterized RAREs are present within *Hox* gene clusters [19,20] and transcription of *Hox* genes is readily upregulated following RA treatments of early vertebrate embryos or *in vitro* cellular models of early embryonic cells [17,21–25

to it. In particular, exogenous RA can promote expression of genes of the *Hoxb* cluster in posterior, but not in anterior NSPCs, which remain resistant to RA-dependent *Hoxb* transcription also during adulthood and ageing. We further show that *Hoxb* chromatin, including key RAREs, is differentially enriched for activating and repressing histone modifications in rostral and caudal NSPCs. In agreement with transcriptional effects, RA treatments are unable to reverse *Hoxb* chromatin repression in rostral NSPCs, but can enhance *Hoxb* chromatin activation in caudal NSPCs.

Altogether, these results suggest that intrinsic epigenetic differences at the level of key regionally expressed genes may constrain transcriptional responses to morphogen signalling and safeguard maintenance of rostrocaudal positional identities in NSPCs.

2. Material and methods

2.1. Mouse neural stem/progenitor cell culture

NSPCs derived from mouse E13.5 cerebral cortex or spinal cord and protocols for their *in vitro* culture were previously described [43]. Cortex NSPCs were obtained from the dorsolateral wall of the telencephalon, corresponding to the developing cerebral cortex. Spinal cord NSPCs were derived from spinal cord tissue dissected between the hindbrain/spinal cord boundary and hindlimb buds. Adult or aged SVZ NSPCs were derived from 3-month- or 18-month-old mice, respectively, and cultured *in vitro* as previously described [44]. NSPCs were routinely expanded in T25 flasks (Corning) that were coated with 10 µg ml^{-1} poly-ornithine (Sigma-Aldrich) and 5 µg ml^{-1} laminin (Sigma-Aldrich), using previously described chemically defined media [43,44] supplemented with 20 ng ml^{-1} human recombinant epidermal growth factor (R&D Systems) and 10 ng ml^{-1} human recombinant fibroblast growth factor-basic (Peprotech). Media for embryonic cortex and spinal cord NSPC culture also contained 1 : 100 N2 supplement (Invitrogen), while 1 : 50 B27 supplement minus vitamin A (Invitrogen) was used for adult SVZ NSPC cultures. NSPCs were passaged every 3–5 days using Accutase (Sigma-Aldrich) and usually seeded at a density of 10–20 000 cells cm^{-2}. NSPCs expanded for up to 20 passages *in vitro* since their initial derivations were used for this work.

2.2. Luciferase assays

To detect activation of retinoid signalling in NSPC cultures treated with RA, 2×10^6 embryonic cortex or spinal cord NSPCs were transfected with 2 µg of tk-$(\beta RARE)_2$-luc plasmid, in which expression of firefly luciferase is under control of RAREs [12], and 100 ng of pRL-TK plasmid, which drives constitutive expression of *Renilla* luciferase as a control for transfection efficiency [45]. NSPC transfection was carried out using an Amaxa mouse neural stem cell Nucleofector kit (Lonza) on an Amaxa Nucleofector device (Lonza). Transfected cells were seeded in one six-well plate (Corning). The day after transfection, half of the wells received fresh media supplemented with all-trans RA (Sigma-Aldrich) diluted from a 25 mM stock in DMSO. Media added to the other half contained equal volumes of DMSO. After 24 h of treatment, cells were harvested and reporter expression levels were measured using the dual-luciferase reporter assay system (Promega) on a GloMax multi+ detection device (Promega) as previously described [45].

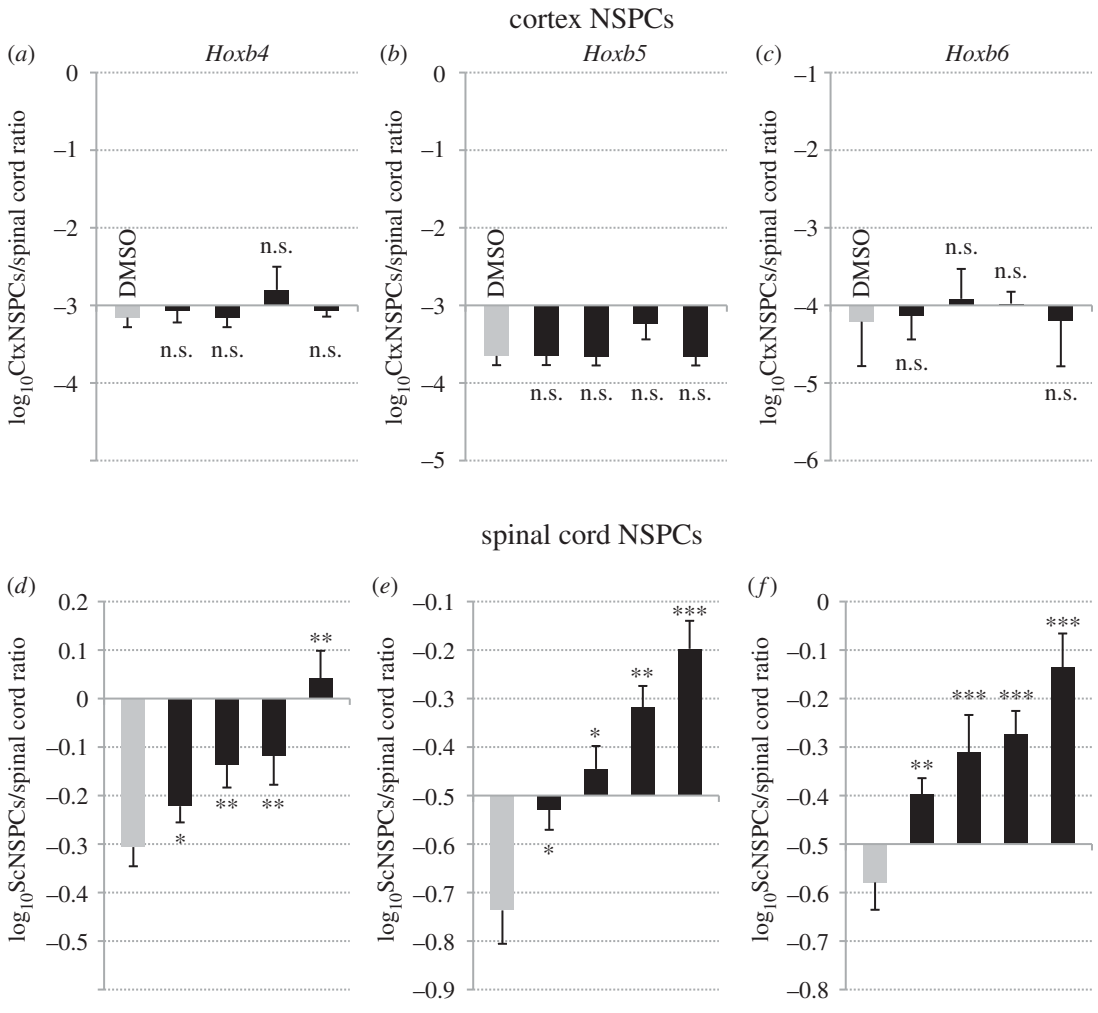
2.3. Real-time RT-PCR

For gene expression analysis by real-time RT-PCR in RA-treated cultures, embryonic cortex or spinal cord NSPCs were seeded in six-well plates (Corning) at a density of 10 000 cells cm^{-2}. Adult SVZ NSPCs were instead seeded in T25 flasks at the same density. After 24 h, cultures received fresh media containing appropriate concentrations of RA or DMSO solvent. Media supplemented with RA or DMSO were again replaced the next day and cultures were harvested for molecular analysis after 48 h of treatment.

Total RNA was extracted from frozen cell pellets using the Qiagen RNeasy mini kit and quantified with a NanoDrop 2000 (Thermo Scientific). For real-time RT-PCR, RNA was reverse-transcribed using the Qiagen QuantiTect reverse transcription kit and amplified on a Rotor-Gene Q (Qiagen), using the Qiagen QuantiFast SYBR Green PCR kit. Primers for real-time RT-PCR were either purchased from Qiagen or designed using PRIMER3 (http://bioinfo.ut.ee/primer3/). Primer sequences selected with PRIMER3 are listed in the electronic supplementary material, table S1. Relative gene expression levels in different samples were determined with the built-in comparative quantitation method [46], using *Eef1a1* or *Rpl19* as reference genes, with similar results. Statistical analysis of experimental data was carried out using the Microsoft EXCEL software.

2.4. Chromatin immunoprecipitation

For detection of histone H3 modifications by chromatin immunoprecipitation (ChIP), NSPCs were seeded in 100 mm plates at a density of 12–18 000 cells cm^{-2}, until cultures reached approximately 80% confluence. Usually, 10–12 $\times$ 10^6 cells for each experimental sample were used in the following steps. At the time of harvesting, cultures were rinsed with PBS, followed by cross-linking with 1% formaldehyde in PBS for 10 min and quenching with 125 mM glycine for 5 min. Cells were then washed with PBS, harvested with a cell scraper in PBS, centrifuged and lysed in lysis buffer (5 mM PIPES, 85 mM KCl, 0.5% NP-40) for 20 min on ice. This was followed by centrifugation, pellet resuspension in shearing buffer (50 mM Tris (pH 8.1), 10 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate), sonication to an approximate size of 250–500 bps and pre-clearing using Dynabeads-protein G (Invitrogen). The protein content of


cortex NSPCs
(a)
Hoxb4
(b)
Hoxb5
(c)
Hoxb6
log10CtxNSPCs/spinal cord ratio
DMSO
n.s.
spinal cord NSPCs
(d)
(e)
(f)
log10ScNSPCs/spinal cord ratio
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cortex NSPCs

spinal cord NSPCs

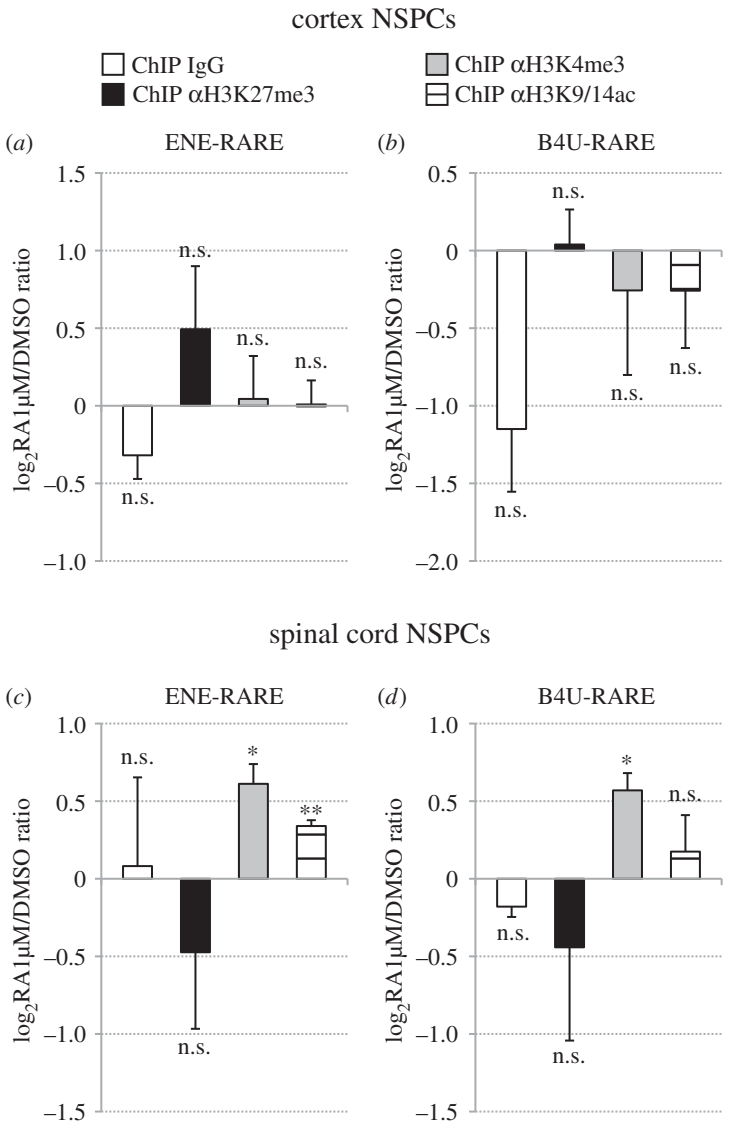
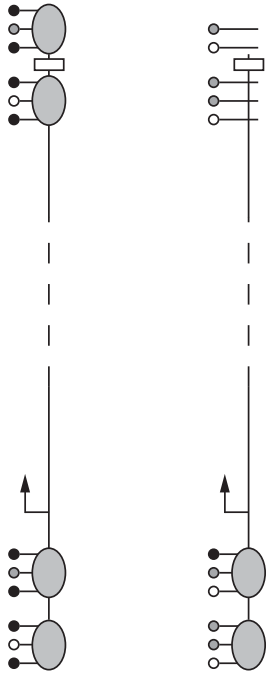


Fig. e ,. Retinoid signalling promotes H3K4me3 and H3K9/14ac deposition in *D6(u12rnxb)]TJ /F4 1 Tf 1.7562 0 TD .0001 Tc [(R)-H K me , H K35me aH K /6 aclevelD6(u12rnxb)]TJ /F4 1 Tf 1.6225 0 TD .1128 Tc [(R)106.2(A)112.4(R)112.6(E)112.7(*
NSPCs that were treated with either DMSO o 1 μM RA for 48 h, using control (IgG), ati-H3K27me3 (αH3K27me3), K4me3) o ati-H3K9/14ac (αH3K9/14ac) atibodies. Pimer pairs used for re PCR target ENE-RARE (a,c) A signi□cant increase in H3K4me3 and H3K9/K14ac levels is detectable in RA-treate spinal cod NSPCselative to es, while H3K27me3 leve are not signi□cantly a□ected. H3K4me3, H3K27me3 a H3K9/K14ac levels do not show es following RA treatments of cote NSPCs. No signi□cant changes between RA-treated and DMSO-eated samples cotro ChIP (ChIP IgG). Results are swn as the mean o the log $_2$-transformed ratio between RA-treated and DMSO-treated NSPCs in three to four biological replicates, following normalization to the reference ao Erro bars sw s.e.m. *$p \leq 0.05$; * $p < 0.01$; n.s., non-signi□cant ($p > 0.05$) acoding to a two-tailed Student's *t*-test performed between RA-treated and DMSO-treted samples.

and ENE-RARE, B4U-RARE and D (figure 3*d–f*). By contrast, the H3K4me3 and H marks we significantly enriched i the same genomic sequences of spinal cord NSPCs (figure 3*a–f*). No significant diferences b cortex and spinal cord NSPCs were detected when samples were immunoprecipitated with a control IgG (figure 3*a–f*). Since



4. Discussion

Research on both pluripotent stem cells and tissue-specific stem cells has made significant headway over the last two decades, raising increasing expectations over their use in regenerative medicine in the near future. As far as the CNS is concerned, it is hoped that NSPCs derived from the embryonic or the adult CNS and/or by directed differentiation of pluripotent stem cells *in vitro* may be employed to repair irreversible damage to the neural tissue caused by injury or disease [52,53]. In either case, a key question

cells, *Hox* gene chromatin features opposite epigenetic marks. Whereas deposition of the repressive mark H3K27me3 was found throughout *Hox* clusters, focal deposition of the activating marks H3K4me3 and H3K9/14ac was observed at the level of *Hox* gene transcription start sites [23,24,57]. Whether this bivalent epigenetic configuration is also a defining feature of *Hox* loci in pluripotent cells *in vivo* remains currently unclear [58]. Remarkably, however, RA treatments during ESC differentiation towards the neural lineage were able to dramatically reshape the distribution of these epigenetic marks, causing H3K27me3 erasure across *Hox* clusters and increased levels and spread of H3K4me3 and H3K9/14ac deposition at *Hox* regulatory regions [23–25]. Supporting the importance of these epigenetic modifications in *Hox* gene regulation, landmark studies showed that, in forebrain explants dissected from E10.5 mouse embryos, *Hox* clusters displayed increased levels of H3K27me3 deposition in comparison to ESCs and were devoid of H3K4me3 marks [57,59]. By contrast, in tissue samples dissected from caudal embryonic regions between E8.5 and E10.5, sequential activation of *Hox* genes from the 3' end to the 5' end of the cluster was accompanied by progressive erasure of H3K27me3 and deposition of H3K4me3 and H3K9/14ac along the same direction [

patterning [54]. We propose that NSPC *in vitro*