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Expression and cellular localization of the transcription factor NeuroD1 in the developing and adult rat pineal gland

Abstract: Circadian rhythms govern many aspects of mammalian physiology. The daily pattern of melatonin synthesis and secretion is one of the classic examples of circadian oscillations. It is mediated by a class of neuroendocrine cells known as pinealocytes which are not yet fully defined. An established method to evaluate functional and cytological characters is through the expression of lineage-specific transcriptional regulators. NeuroD1 is a basic helix-loop-helix transcription factor involved in the specification and maintenance of both endocrine and neuronal phenotypes. We have previously described developmental and adult regulation of NeuroD1 mRNA in the rodent pineal gland. However, the transcript levels were not influenced by the elimination of sympathetic input, suggesting that any rhythmicity of NeuroD1 might be found downstream of transcription. Here, we describe NeuroD1 protein expression and cellular localization in the rat pineal gland during development and the daily cycle. In embryonic and perinatal stages, protein expression follows the mRNA pattern and is predominantly nuclear. Thereafter, NeuroD1 is mostly found in pinealocyte nuclei in the early part of the night and in cytoplasm during the day, a rhythm maintained into adulthood. Additionally, nocturnal nuclear NeuroD1 levels are reduced after sympathetic disruption, an effect mimicked by the in vivo administration of α- and β-adrenoceptor blockers. NeuroD1 phosphorylation at two sites, Ser^{274} and Ser^{336}, associates with nuclear localization in pinealocytes. These data suggest that NeuroD1 influences pineal phenotype both during development and adulthood, in an autonomic and phosphorylation-dependent manner.

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Introduction

The pineal gland is a conserved component of the circadian timing system in vertebrates [1, 2]. It converts photoperiodic information into a circadian rhythm of melatonin synthesis and secretion. The pineal gland develops from an evagination of the dorsal diencephalic roof that begins in rat around embryonic day 15 (E15). From there, proliferation, differentiation, and maturation processes convert the pineal primordium into a structure composed mainly of pinealocytes.

In recent years, several transcription factors responsible for the establishment and maintenance of the pineal phenotype have been identified. The network dynamics and the cellular and molecular mechanisms involved, however, have not yet been fully elucidated. Among these transcriptional regulators, those encoded by homeobox genes are thought to work in an orchestrated manner in the developing and adult rodent pineal gland [3]. Some members of the paired box (Pax), orthodenticle (Otx), and LIM homeobox (Lhx) gene families are essential for normal pineal development, including Pax6, Otx2, and Lhx9 [4–11]. Other homeobox genes like *Crx* (cone-rod homeobox) were found to be nonessential for pineal phenotype, although they might mediate tissue-specific gene expression [12–15]. Crx might modulate pineal homeostasis in a compensational manner with other transcriptional regulators such as Otx2 [16].

Members of the basic helix-loop-helix (bHLH) transcription factor family have also been identified in the rodent pineal gland [17–21]. This is of special interest due to the role of bHLH molecules in generating and maintaining circadian oscillations [22–25]. Our understanding of the precise molecular mechanisms within the cellular circadian clock has advanced significantly in the last decades; little is known, however, about the ontogenetic functions of clock molecules and, conversely, the influence of phenotype determinants in the circadian clock machinery.

The neurogenic differentiation factor 1 (NeuroD1), also known as beta-cell E-box trans-activator 2 (BETA2), has emerged as a potential bHLH link between the ontogenetic and circadian pathways. NeuroD1 was first reported as a converter of *Xenopus* ectoderm into neurons and as a key transactivator of the insulin gene [26, 27]. It is widely accepted that NeuroD1 modulates terminal differentiation and function of defined endocrine and neuronal cell types

via heterodimerization with promiscuous E proteins such as E12/E47 and binding to E-boxes present in the regulatory regions of genes expressed in a tissue-specific manner [27–33].

Retina and pineal gland, two organs thought to evolve from a common photodetecting ancestor, express NeuroD1 [1, 34–40]. While NeuroD1 is essential for differentiation and survival of mouse retinal photoreceptors, pinealocytes survive in the absence of this bHLH but with an affected transcriptome [39, 40]. Global gene expression analyses of two knockout (KO) mouse models revealed potential NeuroD1 target genes in both tissues; these genes are linked to transcription, phototransduction, calcium signaling, and protein folding, among other mechanisms. The clock gene *Per3* was one of the down-regulated genes identified in pineal glands from the Cre/loxP NeuroD1 conditional KO mouse, suggesting a regulatory role of NeuroD1 in the clock machinery [40]. On the other hand, studies of the neurogenic potential of adult rat neural stem/progenitor cells (NSPCs) from the lateral subventricular zone (SVZ) suggested that NeuroD1 is downstream of the clock molecules CLOCK and BMAL1 [41]. In addition, in mice and sheep hypophyseal pars tuberalis, NeuroD1 was identified as part of the transcriptional cascades triggered by melatonin via specific membrane receptors, and downstream of the clock gene *Cry1* [42, 43].

To gain further insight into the function of NeuroD1 in the rodent pineal gland, we characterized NeuroD1 protein dynamics. Here, we report for first time the ontogenetic and daily patterns of NeuroD1 protein, the influence of the sympathetic innervation in NeuroD1 subcellular localization, and NeuroD1 phosphorylation state in the rat pineal gland.

Materials and methods

Animals

All animal experiments and treatments were performed in accordance with the National Institutes of Health's Guide for Care and Use of Laboratory Animals and the Animal Research: Reporting in Vivo Experiments (ARRIVE) Guidelines. All the animal procedures presented here were also approved by the Institutional Animal Care and Use Committee, School of Medicine, National University of Cuyo, Mendoza, Argentina. Wistar rats were housed under a 12:12 light–dark (L:D) cycle with lights turned on at *Zeitgeber* time (ZT) 0, and food and water ad libitum. Male rats were used except for the embryonic series for which both male and female embryos from timed pregnant mothers were processed. Animals were sacrificed by decapitation after ketamine/xylazine (50 and 5 mg/kg of body weight, respectively) anesthesia or hypothermia by immersion in wet ice according to age. Daytime tissues were collected at ZT6 at the following developmental ages: embryonic day (E) 15, 16, 17, 18, and 19, and postnatal day (P) 3, 10, and 90; at night, samples from P3, P10, and P90 rats were obtained under dim red light at ZT14 (early night), ZT18 (middle of the night), and ZT22 (late night). Samples were immediately processed for immunohistochemistry (IHC) or kept frozen at −80°C until their use for Western blot (WB). Removal of superior cervical ganglia (SCGx) was performed according to the procedure described in detail by Savastano et al. [44]. Control animals underwent placebo (sham) surgery. In both groups, samples were collected 3 wk after surgery at ZT6 and ZT14.

In vivo administration of α- and β-adrenoceptor antagonists

As an independent method of sympathetic disruption, male adult rats were injected intraperitoneally (i.p.) with prazosin and/or propranolol (Sigma, St. Louis, MO, USA), and antagonists of α_1- and β-adrenergic receptors, respectively [45]. The goal of this procedure was to study the potential involvement of specific receptors in the effect of the nocturnal endogenous norepinephrine on the nuclear–cytoplasmic partitioning of NeuroD1 protein. Doses of 1 mg/kg of body weight of each antagonist or a 1:1 mixture were applied at ZT11, to give the drugs time to reach their target before the lights were turned off at ZT12. A control group was injected with vehicle alone. Animals were sacrificed as described above at ZT14 (3 hr after injection). Pineal glands were collected and processed for immunohistochemical analysis.

Immunohistochemistry

Samples for immunostaining were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4°C. Entire E15 embryos, whole E16 heads, and adult pineal glands were fixed by immersion; whole brains including pineal glands and cerebella from late embryos and neonatal rats were dissected after transcardial perfusion with the same fixative mixture. After fixation, the organs were washed three times in PBS, dehydrated in increasing concentrations of ethanol (50%, 70%, 80%, 96%, and 100%), washed twice in xylene, and included in Histoplast (Biopack, Bs. As., Argentina). Incubation times in the different solutions varied with tissue size. Three- to ten-micrometer sections from fixed samples were cut using a Microm HM-325 microtome (Thermo Fisher Scientific Inc., Waltham, MA, USA). All the immunohistochemical procedures were performed as previously described [44, 46]. Sections were stained with the following primary antisera: rabbit polyclonal anti-NeuroD1 (ND1), DCK6300, N-terminal epitope: 13 aa: MTKSYSESGLMGE (NeuroD1 protein: 357 aa, NP_062091.1), provided by Dr. D.C. Klein (NIH, USA), dilution 1:50; goat anti-NeuroD1, sc-1084 (N19, N-terminus), Santa Cruz Biotechnology Inc. (Dallas, TX, USA), dilution 1:25; mouse monoclonal antivimentin (VIM), V6630, Sigma, dilution 1:200; and mouse anti-phosphoSer^{10}-histone H3 (pSer^{10}-H3), ab14955, Abcam (Cambridge, MA, USA), dilution 1:100. The secondary antisera included anti-rabbit conjugated with Alexa Fluor 488 and anti-mouse labeled with the Cy3 fluorophore, and biotinylated antibodies, Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA) and Vector Laboratories Inc. (Burlingame, CA, USA), dilution 1:300. When it was required, fluorescein- and horseradish peroxidase (HRP)-conjugated streptavidins

were used, Vector Laboratories Inc., dilution 1:300. The enzyme HRP was detected using 3,3′-diaminobenzidine (DAB; Sigma) as substrate. After immunolabeling, sections were counterstained with hematoxylin (H) or mounted in the presence of propidium iodide (PI; Sigma) diluted in a mix of propyl gallate/PBS/glycerol. The sections were examined using an Olympus FluoView FV-1000 confocal microscope (Olympus America Inc., Center Valley, PA, USA) and Nikon 80I microscope (Nikon Instruments Inc., Melville, NY, USA); images were processed with MacBiophotonic ImageJ and edited with Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA, USA).

Cell counting

For quantification of total and NeuroD1-positive pinealocyte nuclei, sections from sham and SCGx pineal glands collected at ZT6 and ZT14 were processed for IHC using DCK6300 as primary antibody and fluorescein and PI as specific and general nuclear dyes, respectively. Images were captured with the Olympus FluoView FV-1000 confocal microscope using a 60× objective and digitalized with MacBiophotonic ImageJ. Three pineal glands per group and nine images per animal were used. After a 2× magnification of each 60× image, the numbers of total and NeuroD1-positive pinealocyte nuclei were counted in an area of 4×10^{-3} mm^2. Pinealocytes were easily distinguished from interstitial cells because of the nuclear size, chromatin aspect, and presence of multiple nucleoli. A pinealocyte nucleus was considered positive for NeuroD1 when the immunoreactivity was homogeneously distributed in the nuclear area, and the fluorescence levels were higher than those in the cytoplasm.

Western blot analysis

Total proteins from frozen adult pineal gland pools (10–15 glands per pool), cerebella, and pancreas were partitioned into nuclear and cytoplasmic fractions using the CelLytic NuCLEAR Extraction Kit (Sigma) according to the manufacturer's protocol. Lysis and extraction buffers were supplemented with the reducing agent dithiothreitol (DTT; final concentration: 1 mM), protease inhibitor cocktail [diluted from 100× stock solution stored at −20°C, composition: 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatin A, bestatin, leupeptin, aprotinin, and trans-epoxysuccinyl-L-leucyl-amido(4-guanidino)-butane (E-64)], and the phosphatase inhibitor sodium fluoride (NaF; final concentration: 10 mM) [46]. Total protein concentrations were estimated with Bradford reagent (Bio-Rad Laboratories Inc., Hercules, CA, USA) using bovine serum albumin (Sigma) as the standard protein. Nuclear and cytoplasmic samples from sham and SCGx pineal gland pools were collected at ZT14, and positive control tissues were used to study NeuroD1 and its post-translational modifications. Proteins (80 μg per lane for total NeuroD1, 40 μg per lane for NeuroD1 phosphorylated forms) were resolved by 10% SDS–polyacrylamide gel electrophoresis, transferred to PVDF membranes by electroblotting and incubated with blocking solution (10% w/v low-fat milk powder in wash buffer: PBS with 0.05% Tween-20) for 1 hr at RT. Subsequently, the membranes were rinsed three times with wash buffer for 10 min each and incubated overnight at 4°C with the primary antiserum diluted in blocking solution (sc-1084, dilution 1:5000; DCK6300, dilution 1:3000) or in wash buffer [rabbit polyclonal anti-phosphoSer^{274}-NeuroD1 (pSer^{274}-ND1), ab78900, Abcam, dilution 1:5000; rabbit polyclonal anti-phosphoSer^{336}-NeuroD1 (pSer^{336}-ND1) provided by Dr. A. Bonni (Harvard Medical School, USA) [47], dilution 1:5000; rabbit polyclonal anti-actin, A 2066, Sigma, dilution 1:5000; rabbit polyclonal anti-histone H3 (H3), 07-690, Upstate (EMD Millipore, Billerica, MA, USA), dilution 1:10,000]. The membranes were incubated consecutively with the corresponding biotinylated secondary antiserum and with HRP–streptavidin (Vector Laboratories Inc.) for 1 hr at RT each, both diluted in wash buffer (1:50,000). Protein bands were visualized with the LAS-4000 system (Fujifilm) after a chemiluminescent reaction using a 1:1 mixture of solution 1 (20 mM Tris-HCl pH 8.5; 2.5 mM luminol, Sigma; 0.4 mM coumaric acid, Sigma) and solution 2 (10 mM Tris-HCl pH 8.5; 0.02% H_2O_2, Sigma). Histone H3 and actin were used as loading controls for nuclear and cytoplasmic extracts, respectively [46]. Optical density (OD) of target protein bands from three independent experiments was determined from the LAS-4000 files using MacBiophotonic ImageJ software. Final values were expressed as the ratio of NeuroD1/histone H3 in the nuclear fraction and NeuroD1/actin in the cytoplasmic fraction.

Statistical analysis

Data, expressed as mean ± S.E.M., were analyzed using PRISM 5 (GraphPad Software Inc., La Jolla, CA, USA). Statistical differences were determined by two-tailed Student's *t*-test. $P < 0.05$ was considered significant.

Results

To validate the specificity of DCK6300 via IHC, we used developing cerebella from P3 and P10 rats that contain NeuroD1-positive neurons. Preabsorption of the serum with the corresponding antigenic peptide and omission of the primary antibody were included as negative controls. In the cerebellar cortex, NeuroD1 expression is known to correlate well with the genesis and maintenance of glutamatergic granular layer interneurons [39, 46]. We observed that DCK6300 was able to recognize not only progenitor cells in the external germinative/granular layer (EGL) but also migrating cells with spindle-shaped nuclei in the molecular layer (ML) and more mature interneurons in their final destination, the internal granular layer (IGL) (Fig. S1A–I). As expected for a discriminatory antibody, DCK6300 did not react with Purkinje cells and other cerebellar NeuroD1-negative cells. Similar results were obtained with the anti-NeuroD1 antibody N19 (data not shown) [46]. In addition, a band of around 50 kDa was detected by DCK6300 via WB, mainly in the nuclear protein fraction from rat cerebellum (Fig. S1J). We previously reported that NeuroD1 mRNA is highly abundant throughout rat pineal gland development from embryonic

stages to adulthood, that its levels do not appear to be influenced by sympathetic neural input, and that in the absence of *NeuroD1*, the mouse pineal gland transcriptome is affected [39, 40]. To gain a better understanding of NeuroD1 function, and taking advantage of the specificity of the novel DCK6300 antibody, we characterized for the first time NeuroD1 protein dynamics during the entire ontogeny of the rat pineal gland. In rat embryos, NeuroD1 was found mainly in the nuclei of pinealocyte precursor cells. NeuroD1 expression together with the presence of the intermediate filament protein vimentin allowed us to follow the organogenesis of the pineal gland from the dorsal diencephalic evagination to the mature globular structure. Staining for both proteins in the embryonic period revealed normal precursor cell rearrangements from the radial distribution at earlier stages (Fig. 1A–H) to a rosette-like pattern in late gestation (Fig. 1I–P). The high levels of nuclear NeuroD1 in the prenatal period when most of the pinealocyte precursors divide for the last time before differentiation [48]

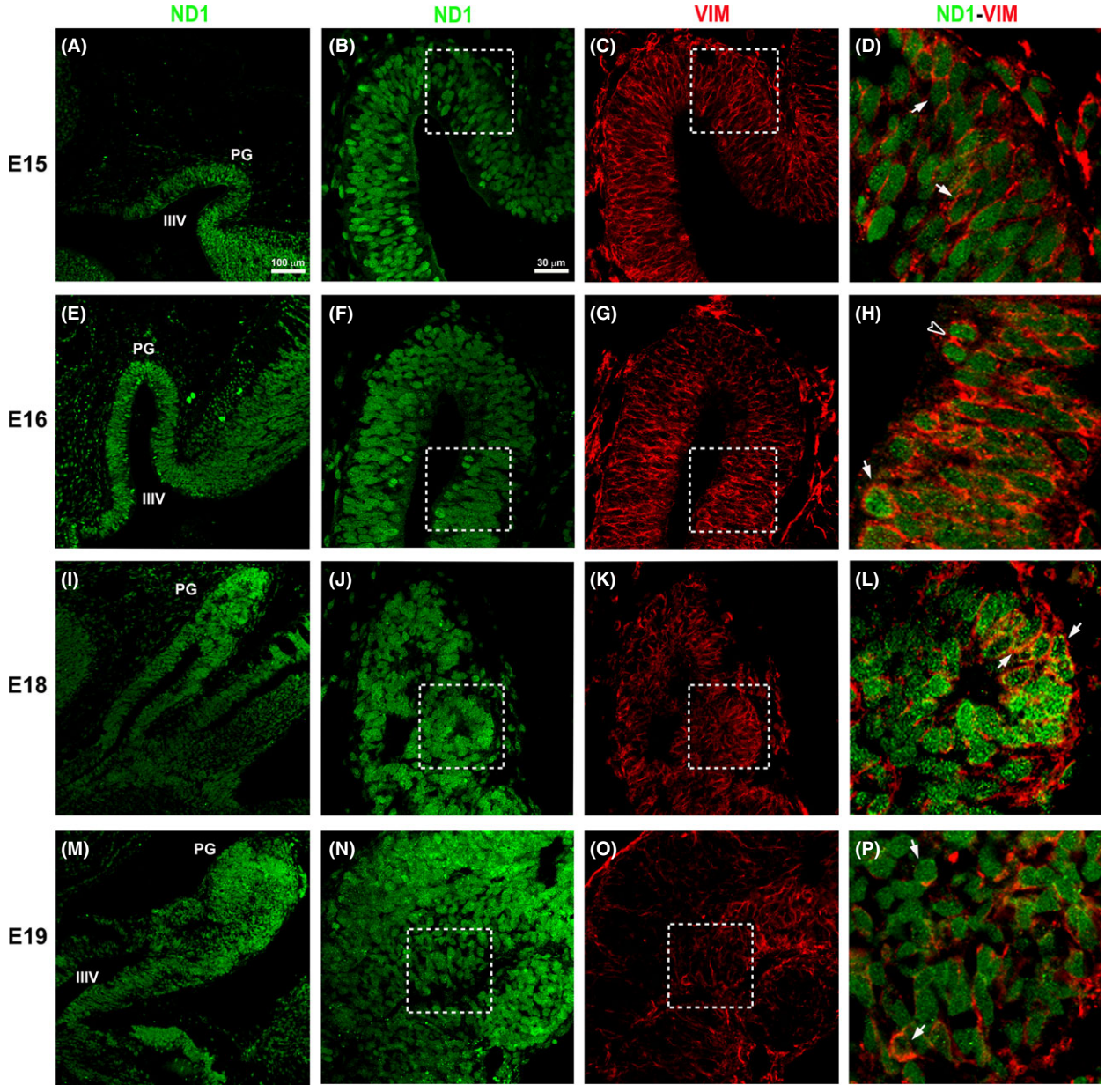

Fig. 1. NeuroD1 protein is expressed in the embryonic rat pineal gland. (A, B, E, F, I, J, M, N) Immunolabeling for the bHLH transcription factor NeuroD1 (ND1, green, Alexa Fluor 488) at embryonic days (E) 15, 16, 18, and 19, using the anti-NeuroD1 antibody DCK6300. Both male and female rat embryos were used. (C, G, K, O) Immunoreactivity for the intermediate filament protein vimentin (VIM, red, Cy3). (D, H, L, P) Combined ND1 and VIM immunolabeling. ND1 is nuclear in pinealocyte precursor cells. ND1 and VIM co-expression (white arrows) reveals features of the pineal gland (PG) organogenesis from a dorsal diencephalic evagination (E15) to a globular structure (E19) passing consecutively throughout tubular elongation (E16) and rosette-like formation (E18) stages. Black arrowhead with white borders points to ND1- and VIM-positive daughters cells. IIIV: Third ventricle. (A, E, I, M) 20×; scale bar: 100 μm. (B, C, F, G, J, K, N, O) 60×; scale bar: 30 μm. (D, H, L, P) Digital zooms of the insets shown at 60×. bHLH, basic helix-loop-helix.

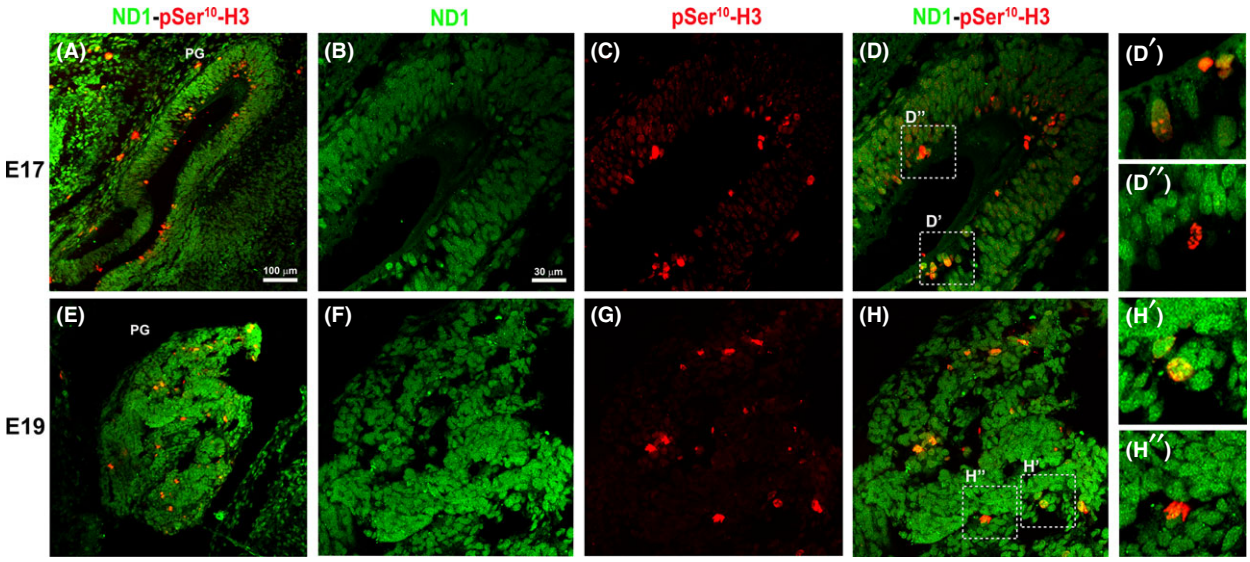

Fig. 2. NeuroD1 is expressed in dividing pinealocyte precursor cells. Combined immunolabeling for NeuroD1 (ND1, green, Alexa Fluor 488) and the mitotic marker phosphoSer[10]-histone H3 (pSer[10]-H3, red, Cy3). (A–D″) ND1 and/or pSer[10]-H3 in the developing pineal gland (PG) at embryonic stage 17 (E17). Mitotic cells are located mainly in the luminal side of the stratified neuroepithelium lining the tubular structure. (E–H″) ND1 and/or pSer[10]-H3 in the globular pineal gland at E19 with proliferating cells randomly distributed. Most of the dividing cells are positive for both ND1 and pSer[10]-H3 (D′ and H′, yellow). Mitotic cells with very low levels of ND1 are shown in D″ and H″. (A, E) 20×; scale bar: 100 μm. (B–D, F–H) 60×; scale bar: 30 μm. (D′–D″, H′–H″) Enlargements of the insets shown in D and H, respectively.

motivated us to question the relationship of this bHLH with the cell cycle in the developing pineal gland. It has not been fully clarified whether NeuroD1 expression in proliferating cells is mostly postmitotic [31]. We addressed this controversy by performing double immunostaining for NeuroD1 and the mitotic marker histone H3 phosphorylated at serine 10, pSer[10]-H3 (Fig. 2) [49]. This strategy confirmed previous results about the abundance of mitotic cells in the prenatal pineal gland [48]. In earlier embryonic phases, highly pSer[10]-H3-positive cells were located in the apical side of the stratified neuroepithelium lining the diencephalic evagination from which pineal gland is derived. In later prenatal developmental stages, the proliferating cells resulted randomly distributed. NeuroD1 levels varied among dividing cells; mitotic cells with a bHLH expression higher than basal predominated in the different stages studied. The ability of the rat pineal gland to synthesize and secrete melatonin in a rhythmic fashion develops during the postnatal period in parallel with the acquisition and maturation of the required enzymatic machinery and responsiveness to adrenergic input, among other regulatory mechanisms [50]. Consequently, we evaluated NeuroD1 distribution in P3 and P10 rats at different *Zeitgeber* times (ZTs). In 3-day-old pups, NeuroD1 protein was detected primarily in the nuclear compartment of pinealoblasts throughout the L:D cycle (Fig. 3A–D; data at ZT6 are shown), a pattern that resembles the one found in the prenatal period. Double immunolabeling for NeuroD1 and vimentin identified embryonic precursor-like cells present in the neonatal pineal gland. At P10, a clear daily pattern in the subcellular localization of NeuroD1 protein was observed with nuclear presence mainly at the beginning of the dark phase (ZT14) (Fig. 3E–L; data at ZT6 and ZT14 are shown). This rhythm was also present in adult pineal glands; however, certain heterogeneity in the nuclear–cytoplasmic partitioning among mature pinealocytes was seen, especially in the light phase (Fig. 4). Norepinephrine released in the pineal parenchyma at night from the nerve endings of sympathetic neurons located in the superior cervical ganglia (SCG) constitutes one of the major regulators of the rhythmic pineal physiology [51]. Based on the observation that NeuroD1 protein was nuclear in the dark phase, adult rats were subjected to chronic bilateral SCGx or sham surgery [44]. As expected for an adrenergic-dependent phenomenon, NeuroD1 nuclear–cytoplasmic partitioning in adult pinealocytes was affected by ganglionectomy (Fig. 5). In SCGx pineal glands collected at ZT14, NeuroD1 was cytoplasmic in the majority of the cells with a perinuclear disposition, a pattern that mimics the one seen in pineal glands from non-operated rats sacrificed at ZT6 (Fig. 4A–E). The number of pinealocytes with NeuroD1-positive nuclei was counted in sham and SCGx animals (Fig. 6A,B). The latter showed a significant reduction in immunoreactive pinealocyte nuclei at ZT14 ($P < 0.001$ versus sham) with numbers comparable with those observed in sham pineal glands at ZT6. The total number of pinealocytes did not vary among the groups (Fig. 6C); therefore, the decrease in NeuroD1-positive nuclei triggered by SCGx was not likely due to cell death. Ganglionectomy effects on NeuroD1 protein dynamics were also analyzed via WB using nuclear and cytoplasmic extracts from sham and SCGx pineal glands (Fig. 7A). Nuclear NeuroD1 levels at ZT14 were diminished after removal of the ganglia, concomitantly with a significant increase in the cytoplasmic fraction ($P < 0.05$ versus sham) (Fig. 7B). Nocturnal norepinephrine activates β_1- and α_1-adrenergic receptors in the pineal gland [52, 53]. To estimate the contribution of these

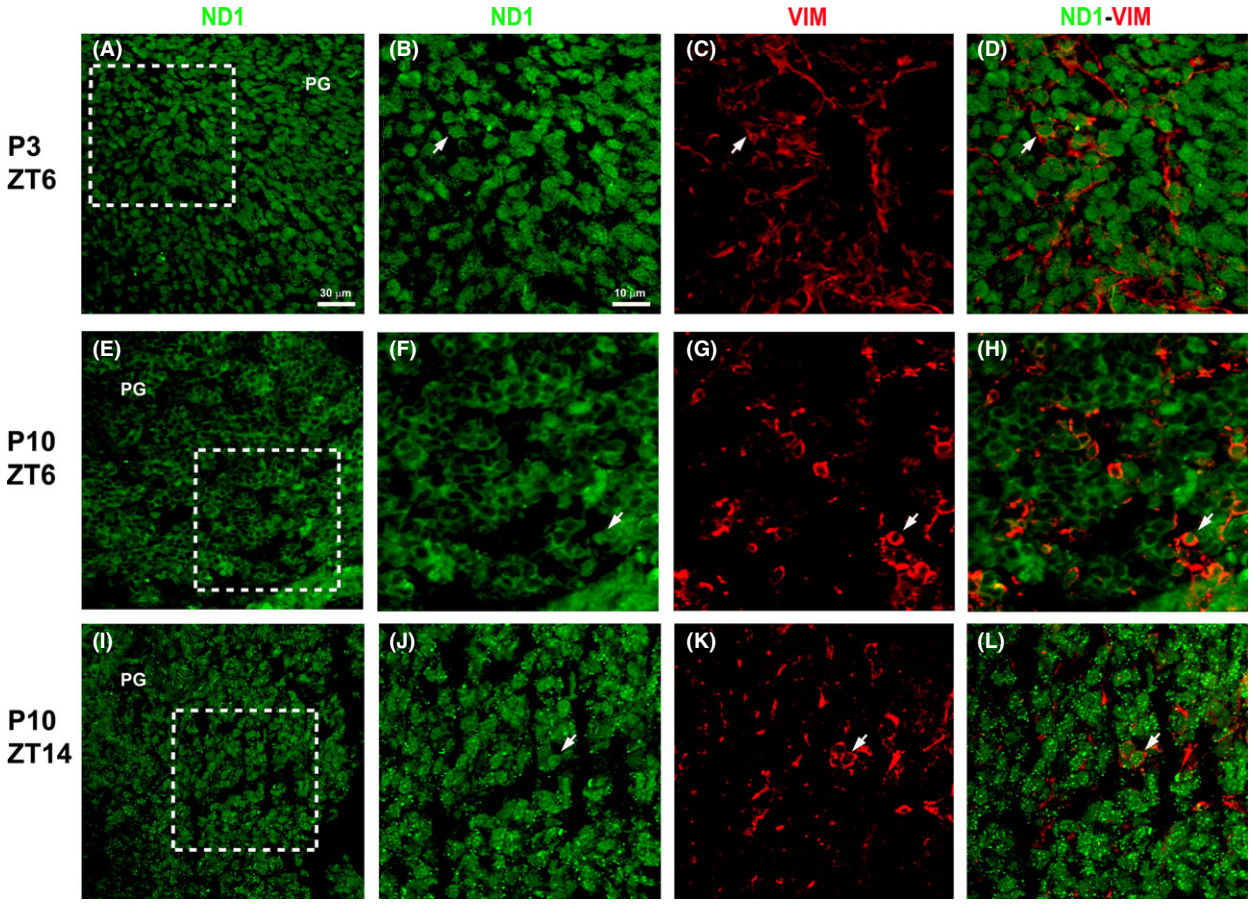

Fig. 3. NeuroD1 protein dynamics in the neonatal rat pineal gland. Immunoreactivity for NeuroD1 (ND1, green, Alexa Fluor 488) and/or vimentin (VIM, red, Cy3) in pineal glands (PG) from 3- and 10-day-old male rats (P3 and P10, respectively). For the later age group, data generated at ZT6 (middle of the light phase) and ZT14 (early night; 2 hr after the lights were turned off) are shown. (A–D) ND1 is nuclear in P3 pinealoblasts at ZT6. (E–L) In P10 pineal glands, ND1 protein exhibits a daily rhythm in subcellular localization. The protein is mainly cytoplasmic during the light phase (ZT6) and nuclear in the early night (ZT14). VIM is still expressed in the neonatal pineal gland; embryonic precursor-like cells enriched in ND1 and VIM are indicated by white arrows at both postnatal ages. (A, E, I) 60×; scale bar: 30 μm. (B–D, F–H, J–L) 2× digital zooms of the insets shown at 60×; scale bar: 10 μm.

receptors and the concomitant activation of specific intracellular signaling cascades on the subcellular partitioning of NeuroD1 protein, we acutely administrated the antagonists prazosin and/or propranolol in vivo as α_1- and β-adrenoceptor blockers, respectively. The i.p. injections were performed at ZT11, 1 hr before the lights were turned off at ZT12. Samples were collected at ZT14 when NeuroD1 protein levels would be expected to reach their highest levels in the nuclear compartment of pinealocytes. Both individual and combined treatments caused a reduction in NeuroD1-positive pinealocyte nuclei densities and in nuclear immunoreactivity intensities (Fig. 8; data with each blocker are shown). While in the pineal glands from vehicle-injected animals NeuroD1 was primarily nuclear at the beginning of the night, the experimental groups showed diffusely immunoreactive nuclei and cytoplasmic levels higher than the ones in controls. NeuroD1 protein has been considered a highly modifiable molecule; post-translational modifications such as phosphorylation have been related to the timing of its nuclear localization and transcriptional functions in a species- and cell type-specific manner [30, 54–56]. The addition of phosphate groups represents a key regulatory event in the rhythmic pineal physiology [51]. We therefore studied the phosphorylation state of NeuroD1 in the adult pineal gland at ZT14 via WB and using specific primary antibodies directed against two serine (Ser) residues at positions 274 and 336. A band of around 50 kDa was identified with both antisera (Fig. 9). While phosphoSer^{274}- and phosphoSer^{336}-NeuroD1 (pSer^{274}-ND1 and pSer^{336}-ND1, respectively) were almost undetectable in the cytoplasmic compartment, the nuclear fractions from pools of pineal glands collected at the beginning of the night resulted enriched in both phosphorylated forms. Pancreas and cerebellum were included as positive controls. The former showed similar levels of both isoforms in the cytoplasmic and nuclear compartments. In cerebellum, the nuclear fraction resulted enriched at least in pSer^{336}-ND1.

Discussion

In this work, we describe the expression pattern of NeuroD1 protein in the developing and adult rat pineal gland. The levels of protein expression correlate well with

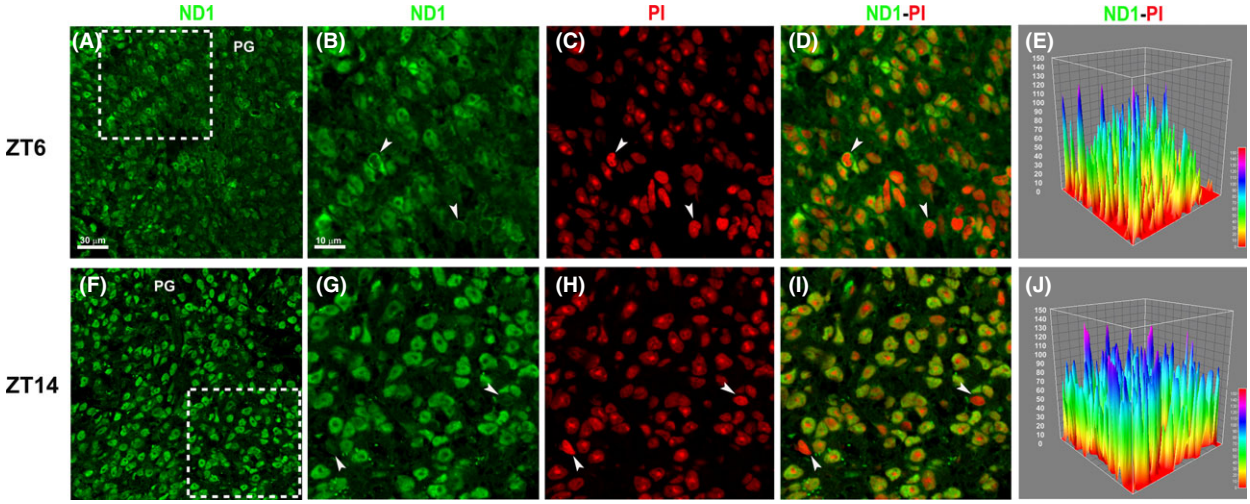

Fig. 4. Daily rhythm in NeuroD1 subcellular localization in adult rat pinealocytes. (A–J) Immunoreactivity for NeuroD1 (ND1, green, fluorescein) and/or nuclear staining with propidium iodide (PI, red) in adult male rat pineal glands (PG) collected at ZT6 and ZT14. While the levels of ND1 in the pinealocyte nuclei are high and relatively homogenous at night, certain heterogeneity among cells is observed at ZT6 being the bHLH mainly cytoplasmic during the light phase. (E, J) Graphical representations of ND1- and PI-stained nuclei densities in D and I, and the range of fluorescence intensities expressed in an arbitrary color scale from 0 to 150 indicated on the right. Each peak represents an individual nucleus. White arrowheads: NeuroD1-negative and PI-positive nuclei as a validation of the discriminatory ability of the antibody DCK6300. (A, F) 60×; scale bar: 30 μm. (B–D, G–I) 2× digital zooms of the insets shown in A and F, respectively; scale bar: 10 μm. bHLH, basic helix-loop-helix.

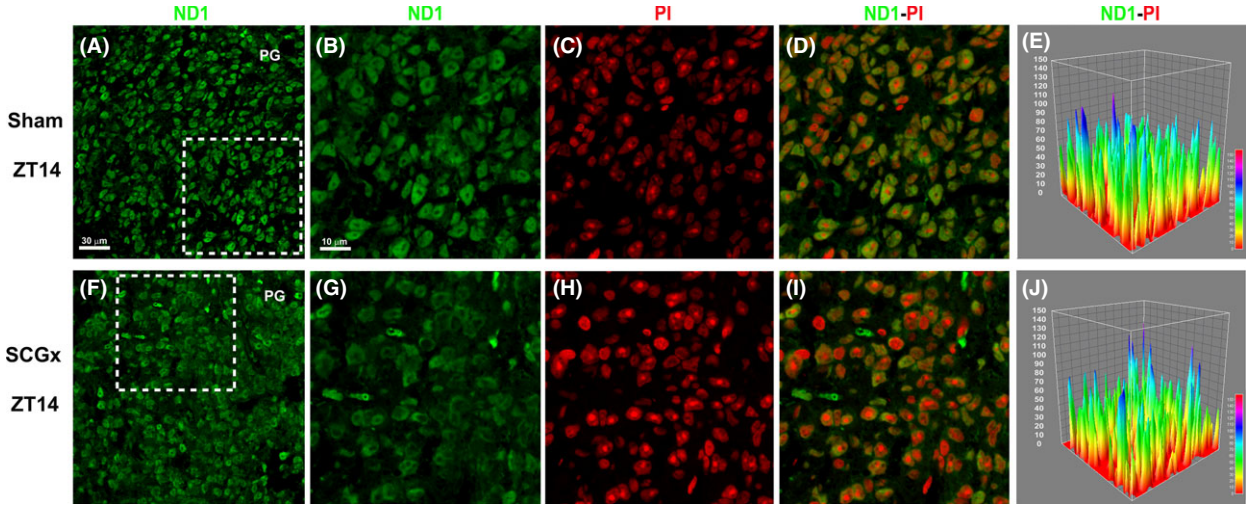

Fig. 5. Influence of the sympathetic innervation in the nuclear–cytoplasmic partitioning of NeuroD1 protein in adult rat pinealocytes. (A–J) Fluorescence immunolabeling for NeuroD1 (ND1, green, fluorescein) and/or nuclear staining with propidium iodide (PI, red) in pineal glands (PG) from adult male rats subjected to chronic bilateral superior cervical ganglionectomy (SCGx) or fake surgery (sham). Samples were collected at ZT14. ND1 localization in sham pineal glands is primarily nuclear. SCGx pinealocytes show cytoplasmic ND1 with preference for the perinuclear region although certain heterogeneity in the subcellular partitioning is observed in this group. (E, J) Graphical representations of ND1- and PI-stained nuclei densities in D and I, and the range of fluorescence intensities expressed in an arbitrary color scale from 0 to 150 indicated on the right. (A, F) 60×; scale bar: 30 μm. (B–D, G–I) 2× magnifications of the insets shown in A and F, respectively; scale bar: 10 μm.

the previously published mRNA profile from the ontogenetic point of view, but its subcellular localization is sensitive to sympathetic neural influence [39]. With the novel anti-NeuroD1 antibody DCK6300, we were able to identify NeuroD1 protein as early as embryonic day 15 (E15), and during all subsequent pineal gland developmental stages. Interestingly, NeuroD1 was primarily nuclear from the time pineal gland formation begins until the sympathetic innervation becomes fully functional around the second week after birth (Figs 1, 2 and 3A–D) [50, 57]. Thereafter, NeuroD1 protein exhibited a daily rhythm in subcellular localization, being present in the pinealocyte nuclei early at night (ZT14) (Figs 3E–L and 4). These results support the hypothesis that NeuroD1 might modulate not only the establishment of the pineal phenotype, but also its maintenance from the juvenile stage onward, and that this late-term influence appears to be rhythmic (Fig. 10).

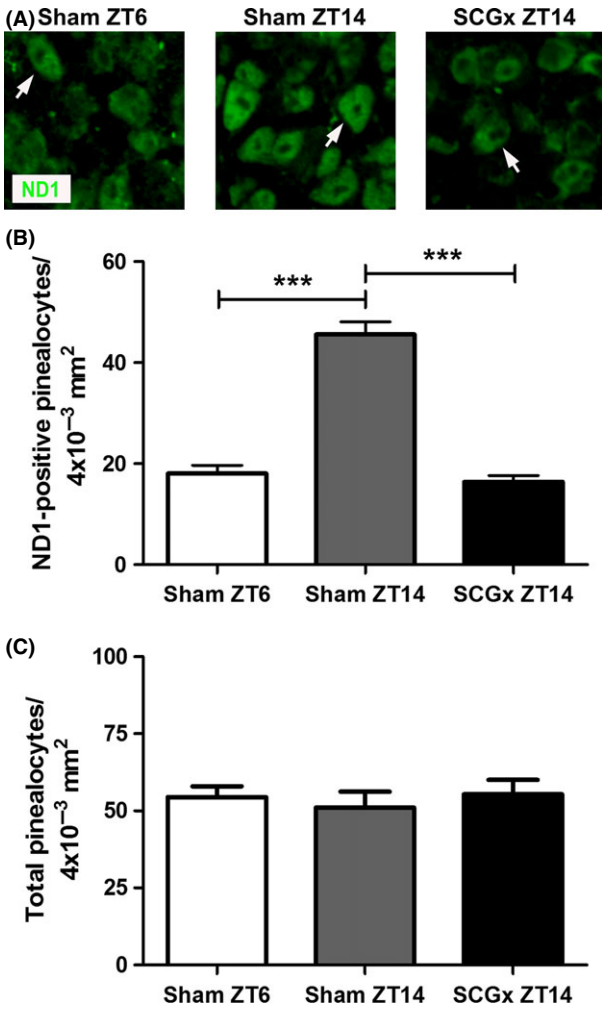

Fig. 6. SCGx effect on the number of NeuroD1-positive pinealocyte nuclei. (A) Representative images of pinealocyte nuclei considered positive for NeuroD1 (ND1, green, fluorescein, white arrows) at ZT6 and ZT14 from sham and SCGx animals. A relatively homogenous nuclear distribution of the immunoreactivity with exception of nucleolar areas and fluorescence levels superior to those in the cytoplasm were taken into account. (B) Quantifications of ND1-positive nuclei. SCGx group shows a significant decrease in the number of immunoreactive nuclei at ZT14; the values are comparable with those in the sham group at ZT6. (C) Total pinealocytes did not vary among the groups. Data are expressed as mean ± S.E.M. in an area of 4×10^{-3} mm^2. Statistics: two-tailed Student's *t*-test; ***$P < 0.001$. SCGx, superior cervical ganglionectomy.

While Pax6, Otx2, and Lhx9 are essential for rodent pineal gland organogenesis, Crx and NeuroD1 are not [6, 8, 9, 11–15, 39, 40]. Two NeuroD1 KO mice, one global and one Cre/loxP-mediated conditional, exhibited pineal gland formation with a relatively normal macrostructure; however, several mRNAs related to transcription, phototransduction, and other signaling cascades were affected [39, 40]. In addition, the daily variations of some transcripts were found to be disrupted in the retina and pineal gland from the conditional KO mouse during adulthood, suggesting an oscillatory role for NeuroD1 in both organs [40]. Based on these data, we can speculate that the modulatory functions of NeuroD1 in the pineal gland might be at least partially compensated by other phenotype determinants when the gene is absent. Compensatory and cooperative mechanisms among bHLH members have been proposed within the retina where the specification and survival of neuronal subtypes are intricately and tightly regulated [58–61]. Cross talk between essential homeobox transcriptional regulators and NeuroD1 in the rodent pineal gland ontogeny can not be excluded. During endocrine pancreas differentiation, for example, Pax6 was identified as both a regulator and a target of NeuroD1, while Pax4, to the best of our knowledge, has only been reported downstream of this bHLH [62, 63]. Based on our observations of early nuclear NeuroD1 presence (Fig. 1) and the precedent of developmentally regulated Pax6 and Pax4 expression in the rodent pineal gland [3, 64, 65], we can propose a similar role for NeuroD1 in this melatonin-producing organ. Our data also revealed that in the neonatal pineal gland, nuclear NeuroD1 is present in a subpopulation of cells (Fig. 3) that resemble those positive for both NeuroD1 and vimentin in the embryonic period (Fig. 1). It would be interesting therefore to investigate whether or not the apparently normal macrostructure of the NeuroD1 KO pineal glands hides altered proportions of cell types as in the mutant retina and whether the nuclear–cytoplasmic distribution of NeuroD1 in the doubly immunolabeled cells is rhythmic as in pinealocytes.

Strikingly, we observed elevated levels of nuclear NeuroD1 in vimentin-positive precursor cells in the highly proliferative prenatal period [48], including in those with a radial rearrangement (Fig. 1). Dividing cells enriched in both NeuroD1 and the mitotic marker pSer^{10}-H3 were clearly identified with a distribution that correlated well with the developmental stages studied (Fig. 2). These results contribute information relevant to the controversy regarding the mitotic and/or postmitotic roles of NeuroD1, which appear to vary across species, developmental phases, and cell types [26, 30, 31, 66–68]. As in the murine cerebellum and hippocampal dentate gyrus, our data suggest that NeuroD1 may regulate not only cellular differentiation but also proliferation within the rat pineal gland where tissue-specific mechanisms might be involved.

The 24-hr dynamics of adult pineal gland biology, including the multistep melatonin biosynthesis, mainly involve adrenergic–cyclic AMP signaling [51]. NeuroD1 mRNA, however, was not found to be influenced by this regulatory pathway [39]. To investigate whether the daily pattern in subcellular localization of the NeuroD1 protein in pinealocytes is under sympathetic neural control, we disrupted the photoneuroendocrine system by chronic bilateral SCG removal (SCGx) (Figs 5–7) and, independently, by acute in vivo administration of adrenoceptor antagonists (Fig. 8). Interestingly, under both treatments, NeuroD1 was retained in the cytoplasm of the vast majority of the pinealocytes at ZT14. These results suggest that norepinephrine released from sympathetic nerve endings during the dark phase influences the nuclear–cytoplasmic partitioning of NeuroD1 via signaling cascades that involve specific membrane receptors (Fig. 10).

NeuroD1 subcellular localization, and therefore its transcriptional activity, was also found to be closely related to

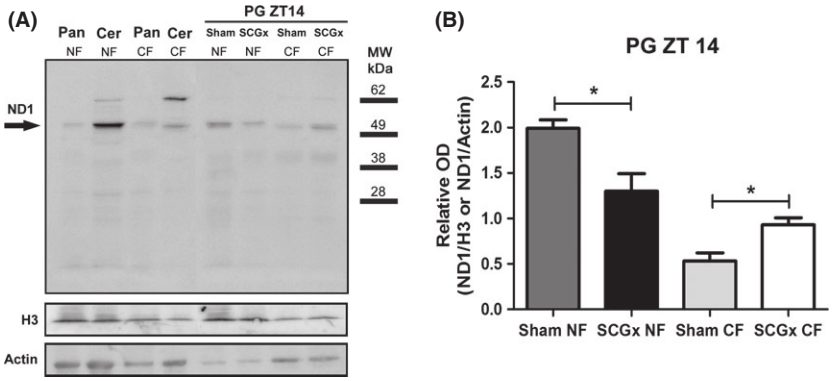

Fig. 7. Nuclear and cytoplasmic NeuroD1 protein levels in SCGx and sham pineal glands. Extract proteins from sham and SCGx pineal glands (PG) collected at ZT14 and cerebellum (Cer) and pancreas (Pan) as positive controls were analyzed for NeuroD1 (ND1), histone H3 (H3) and actin via Western blot (WB). (A) Representative blot of three independent experiments using different pineal gland pools from both groups showing a ND1 band of around 50 kDa (black arrow) with the primary antibody N19. The bands for the loading controls are shown in the bottom. CF, cytoplasmic fraction; MW, molecular weight; NF, nuclear fraction. (B) Quantifications expressed as the mean of the optical density (OD) of ND1 relative to histone H3 in the nuclear fraction or actin in the cytoplasmic fraction, ±S.E.M. Statistics: two-tailed Student's *t*-test; $*P < 0.05$. SCGx, superior cervical ganglionectomy.

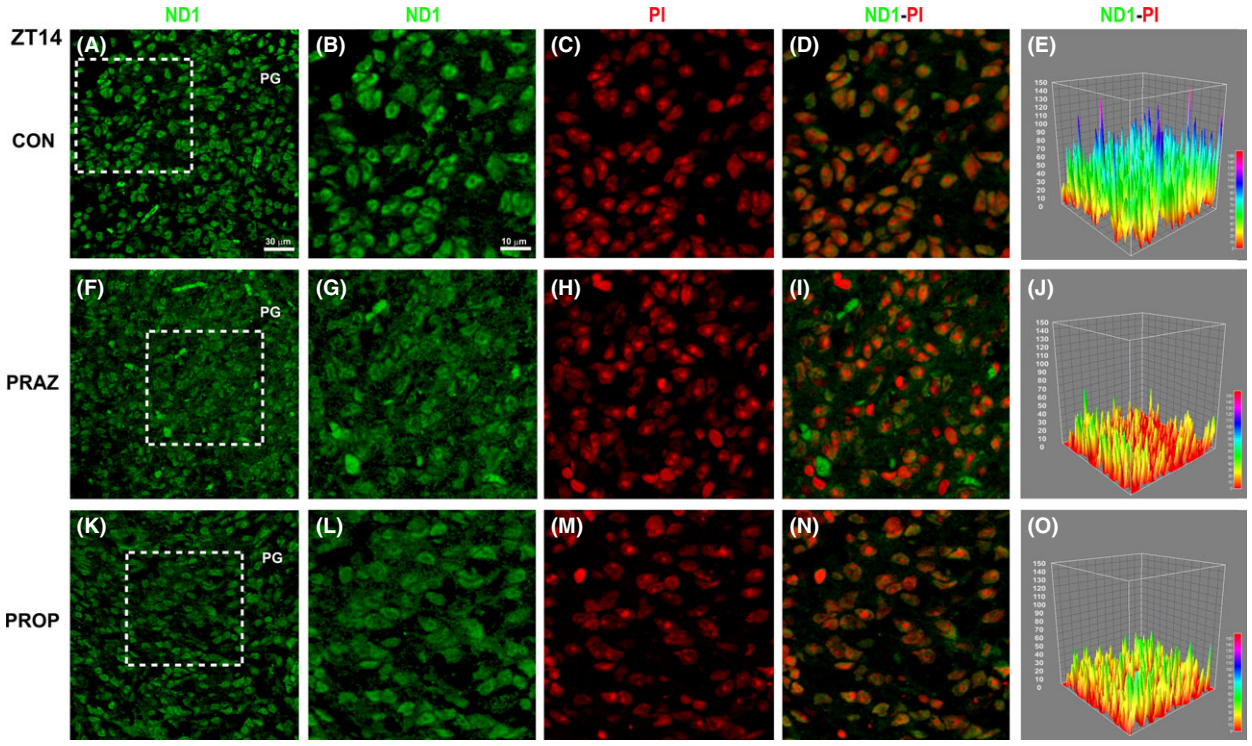

Fig. 8. Influence of α- and β-adrenergic receptors on NeuroD1 nuclear–cytoplasmic partitioning in adult rat pinealocytes. NeuroD1 immunoreactivity (ND1, green, fluorescein) and/or nuclear staining with propidium iodide (PI, red) in pineal glands (PG) from adult male rats treated with vehicle (CON, A–E), the α_1-adrenoceptor antagonist prazosin (PRAZ, 1 mg/kg of body weight, F–J), and the β-adrenoceptor blocker propranolol (PROP, 1 mg/kg of body weight, K–O). (E, J, O) Graphical representations of ND1- and PI-stained nuclei densities in D, I, and N, and the range of fluorescence intensities expressed in an arbitrary color scale from 0 to 150 indicated on the right. Both parameters were affected by antagonist administration; signal intensities were not higher than 70 in the treated PGs. (A, F, K) 60×; scale bar: 30 µm. (B–D, G–I, L–N) 2× enlargements of the insets shown at 60×; scale bar: 10 µm.

cell type-specific physiological functions in other organs such as pancreas and cerebellum. Stimulating MIN6 mouse insulinoma cells with glucose induced insulin gene transactivation, at least in part by facilitating NeuroD1 translocation into the nuclei [54, 69]. Furthermore, neuronal activity promotes nuclear NeuroD1 localization and subsequent dendrite morphogenesis in cerebellar granule cells [47]. The rhythmic nature of nuclear NeuroD1 levels in pinealocytes may explain the altered differential day/night expressions of certain genes in the adult conditional KO mouse [40]. NeuroD1 is not the only pineal phenotype determinant with an oscillatory profile; Otx2, Pax4, Crx, and Lhx4 also

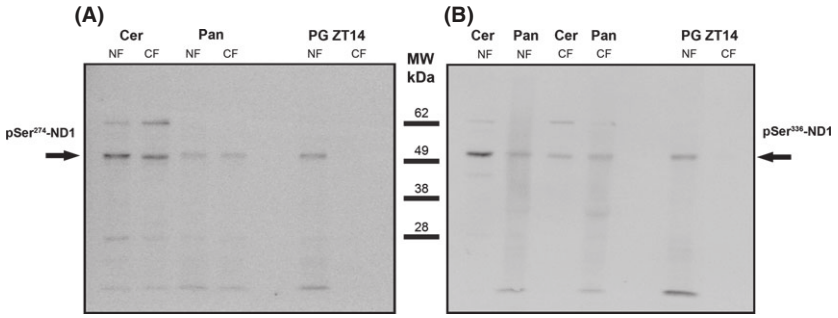

Fig. 9. NeuroD1 is phosphorylated on Ser^{274} and Ser^{336} in the adult rat pineal gland. (A) Immunodetection of phosphoSer^{274}-NeuroD1 ($pSer^{274}$-ND1) via WB and using nuclear (NF) and cytoplasmic (CF) fractions from sham and SCGx pineal glands (PG) at ZT14, and cerebellum (Cer) and pancreas (Pan) as positive controls. (B) Western blotting for phosphoSer^{336}-NeuroD1 ($pSer^{336}$-ND1). A specific band of around 50 kDa is indicated by a black arrow on each blot. Pineal nuclear fractions are enriched in both phosphorylated isoforms. In pancreas, the distribution of the forms is equal in both compartments. In cerebellum, $pSer^{336}$-ND1 seems to predominate in the nuclear fraction. MW, molecular weight; SCGx, superior cervical ganglionectomy.

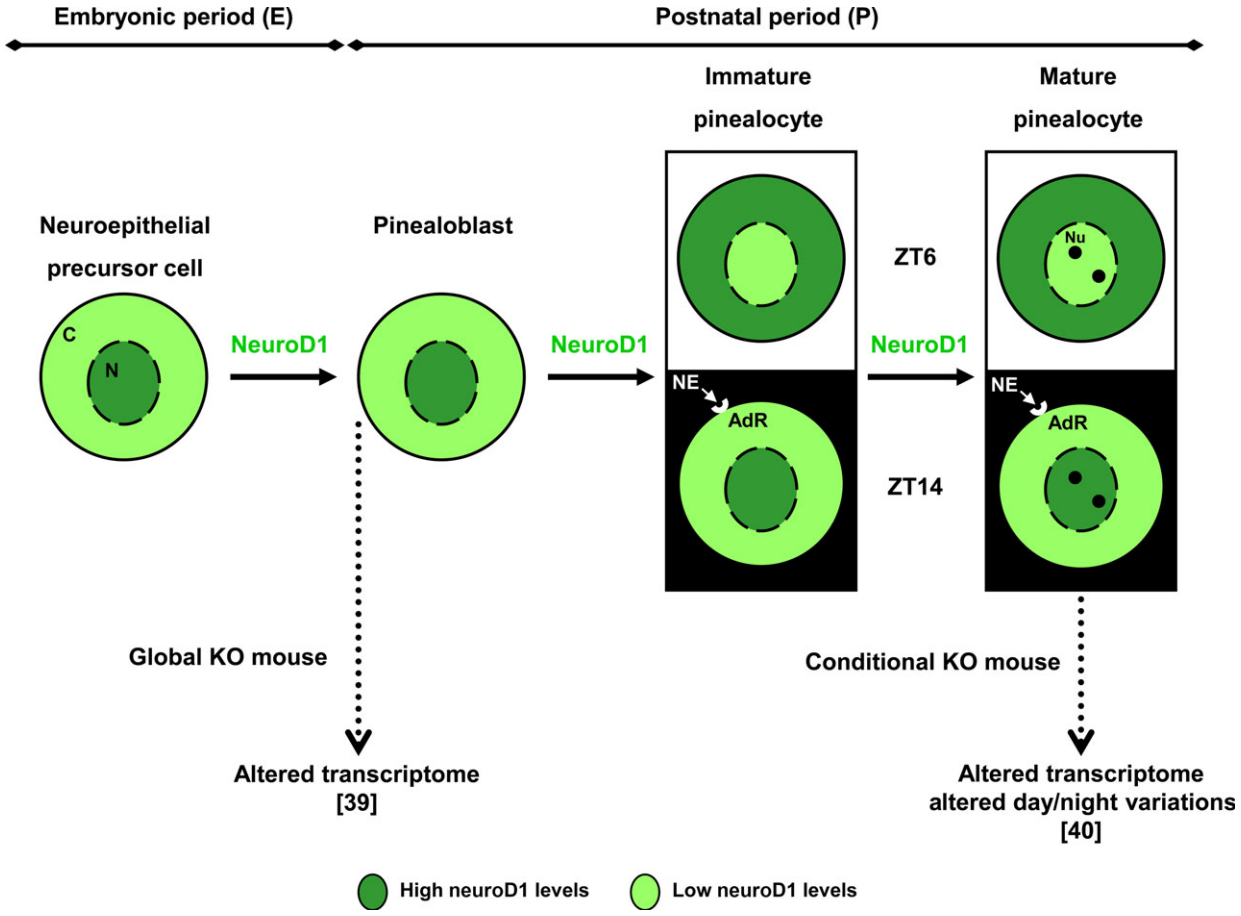

Fig. 10. Schematic model of the evolving modulatory roles of NeuroD1 in the establishment and maintenance of pinealocyte phenotype. NeuroD1 is present during the entire ontogeny of the rat pineal gland. During embryonic and early postnatal stages, when the precursor cells commit to the pinealocyte lineage, the protein has a predominantly nuclear localization that is stable over the light–dark cycle. As the pinealoblast develops into the immature pinealocyte, a daily rhythm in nuclear–cytoplasmic partitioning appears with the protein in the cytoplasm during the light phase (above) and moving into the nucleus in the dark phase (below). This daily oscillation in subcellular localization responds to sympathetic influence. Both the rhythmic partitioning and the autonomic regulation are maintained into adulthood. The absence of *NeuroD1* in two different KO mouse models caused alterations in the pineal gland transcriptome [39, 40]. Although NeuroD1 has been shown not to be essential like Pax6, Otx2, and Lhx9, it may be speculated that it interacts in a compensatory or cooperative manner with other bHLH and/or homeobox transcription factors to modulate pineal gland development and homeostasis. AdR, membrane adrenergic receptors; bHLH: basic helix-loop-helix; C, cytoplasm; KO, knockout; N, nucleus; NE, nocturnal norepinephrine; Nu, nucleolus; ZT, *Zeitgeber* time.

show rhythmic variations at the messenger and/or protein levels [11, 15, 64, 65]. It is likewise reasonable to suspect that different combinations of these factors could orchestrate daily rhythms of pineal-specific genes in addition to their roles in the establishment of pineal phenotype.

NeuroD1 nuclear translocation in insulinoma cells and cerebellar granular neurons was found to be elicited by its post-translational modification, specifically, serine phosphorylation [47, 54]. However, the effect of specific amino acid alterations on the NeuroD1 protein varies across species and cell types, and opposing outcomes have been described [56]. Efficient NeuroD1 nuclear import might also be achieved by synergic heterodimerization with its partner, transcription factor E [70]. In the context of the pineal gland, phosphorylation and dephosphorylation are necessary to maintain an oscillatory physiology. For example, phosphorylation of the transcription factor CREB and the rate-controlling enzyme AA-NAT are required for the nocturnal rise of melatonin; the loss of these phosphate groups contributes at least partially to the decline in hormone synthesis late in the dark phase [71, 72]. We therefore investigated the phosphorylation of pineal NeuroD1 early at night, when it is mainly nuclear. We observed that NeuroD1 protein is phosphorylated on at least two different serine residues, Ser^{274} and Ser^{336} (Fig. 9).

The effect of each individual modification on multiple NeuroD1 characteristics – stability, partnering ability, nuclear–cytoplasmic trafficking, DNA binding, and formation of transcriptionally active complexes – in the pineal environment remains to be investigated. It would also be interesting to examine potential interactions between adrenergic receptor-activated pathways and those involving NeuroD1-modifying enzymes. NeuroD1 Ser^{274} is part of an ERK1/2 site and also of an overlapping GSK3β consensus sequence, enzymes that could mediate stimulatory versus inhibitory effects of NeuroD1 by phosphorylating the same site in different contexts [30, 54–56]. It is likely that members of the mitogen-activated protein kinase (MAPK) family also modify NeuroD1 Ser^{274} in the rodent pineal gland. This kinase family was found to be closely connected to cellular pathways activated by adrenergic receptors in pinealocytes and therefore has been proposed to be involved in the rhythmic biology of the pineal gland [73]. Phosphorylation of Ser^{336} is another important physiological modification of NeuroD1 that has been attributed to neuronal activity-induced CaMKIIα activity in the cerebellum [30, 47]. We are currently investigating the potential involvement of kinases of the Ca^{2+}/calmodulin-dependent protein kinase superfamily [74] in pineal NeuroD1 Ser^{336} modification. Other post-translational modifications and target sites have been described for NeuroD1, each of them with a context-dependent influence [30, 31, 69]. Together these findings suggest that NeuroD1 is highly modifiable by different pathways. The rhythmic influence of NeuroD1 in pineal gland physiology may also be indirect via temporal interactions with the dominant negative HLH inhibitor of differentiation (Id) factors, which dissemble the transcriptionally active complex NeuroD1-E from its target genes. At least Id1 was found to have an oscillatory nature in the rodent pineal gland [75].

In summary, we describe for the first time the ontogenetic and daily pattern of NeuroD1 protein in the rat pineal gland, the influence of sympathetic innervation in its nuclear–cytoplasmic partitioning, and post-translational modifications that could finally modulate NeuroD1 transcriptional activity.

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Author contributions

EMM conceived and designed the experiments. AEC, SGB, LEFA, and LES performed the experiments. AEC, SGB, SIP, and EMM analyzed the data. SIP and EMM contributed reagents/materials/analysis tools. SIP and EMM contributed to the writing of the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Specificity of the anti-NeuroD1 antibody DCK6300.