Neuronal expression of SOX2 is enriched in specific hypothalamic cell groups

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ABSTRACT

The transcription factor SOX2 has many established roles in neural development but is generally considered to have limited activity in the adult brain. As part of a study of neuronal phenotypes in the adult rodent hypothalamus, we have now used immunohistochemical analysis to investigate the expression of SOX2 in the adult rat and mouse hypothalamus. Our analysis has revealed that SOX2 protein is extensively expressed in cells of the suprachiasmatic nucleus (SCN). Co-localization with the nuclear marker proteins NeuN and MeCP2 confirmed SOX2 expression in mature neurons of the rat SCN, and the functional integrity of these SOX2+ neurons was also confirmed by demonstrating co-localization with light-induced EGR1 protein. In addition to the SCN, we have also revealed a population of SOX2+/(NeuN+/ MeCP2+) neurons in the rat periventricular nucleus (PeN). However, in other hypothalamic nuclei such as the supraoptic nucleus (SON) SOX2+ cells were rare. In extra-hypothalamic areas, SOX2+ cells were also scarce although we have confirmed populations of non-neuronal SOX2+ cells in both the rat sub-ventricular zone (SVZ) and sub-granular zone (SGZ) of the hippocampus. In addition, we have identified an extensive, novel population of non-neuronal SOX2+ cells in the rat subfornical organ (SFO). Our findings provide further evidence of 'immature' phenotypes in rodent SCN neurons and, given the extensive expression of SOX2 across these hypothalamic neurons, may identify a common regulatory factor that maintains this unusual neuronal phenotype. Conservation of SCN SOX2 expression in both rat and mouse indicates a functional requirement for this transcription factor that may be integral to the role of these SCN neurons in mediating daily physiological rhythms.

1. INTRODUCTION

SOX2 (SRY (sex determining region Y)-box 2) is a member of the *Sox* family of transcription factors with multiple, well-established roles in the regulation of cell fate during mammalian development (Sarkar and Hochedlinger, 2013). With respect to neuronal development, *Sox2* is involved in both the early establishment of neural lineages (Takemoto et al, 2011) and also the differentiation of specific neuronal subtypes such as GABAergic interneurons (Cavallaro et al, 2008). In general, *Sox2* is most highly expressed during development and expression recedes in the maturing brain; *prima facie* this temporal expression pattern is consistent with the establishment of mature neuronal networks that largely perpetuate for the duration of adult life (Fishell & Heintz, 2013). However, *Sox2* expression is maintained in certain cells of the adult brain: in progenitor cells and astroglia in the adult rat brain (Komitova and Eriksson, 2004), and in minority populations of differentiated neurons as evidenced from transgenic mouse studies (Ferri et al, 2004; Kang and Hebert, 2012). However, adult neuronal expression of SOX2 protein has not been systematically investigated, particularly with respect to the hypothalamus.

We are interested in hypothalamic *Sox2* expression because, in previous studies, we have obtained evidence of 'immature' neuronal phenotypes in one region of the rodent hypothalamus, the suprachiasmatic nucleus (SCN; Geoghegan & Carter, 2008). We showed that populations of neurons in the rat SCN exhibits two unusual characteristics, firstly, expression of the developmental protein doublecortin (DCX) and secondly, a more widespread absence of the mature neuronal marker protein NeuN/Fox-3 (Kim et al, 2009); the latter property is shared by a minority of other extra-hypothalamic populations of adult neurons (Mullen et al, 1992). Recent work in

other laboratories has supported the evidence of immature phenotypes in the SCN although the presence of species/strain differences (Morin et al, 2011; Saaltink et al, 2012) in both cellular distribution and specific developmental molecule suggest that these unusual protein expression patterns may be downstream of a conserved molecular/developmental regulatory mechanism. In recent work we have now investigated the expression of one candidate regulator, namely *Sox2*.

2. METHODS

2.1. Animal Studies

Animal studies were conducted in accordance with the Animal (Scientific Procedures) Act 1986, and Cardiff University ethical review. Male Sprague-Dawley rats and C57BL/6J mice were maintained in a 14:10 light:dark cycle (lights on: 05.00h) in conventional rodent cages (2-5/cage rats; 5-8/cage mice) with standard rodent chow and water freely available. Health status was monitored and assessed by veterinarian consultation if required. For the majority of analyses, rats were killed at young (postnatal day [PND]50; n=5 rats) and old (PND293; n=2 rats) adult stages and mice were killed at 5 months of age (n=2 mice). In additional experiments, the effect of a light pulse during the dark period was investigated by sampling rat brains (PN50) at 01.00h either after a 1h pulse of light (300 lux, n=2) or in the absence of a light pulse (n=2 control rats). A subsequent quantitative analysis of SOX2+ cells in the light pulse paradigm was conducted using parallel-processed tissue from two groups of PN50 rats (n=3 light pulse; n=3 control). Brain tissues were taken for either immunohistochemical or immunoblot analysis (see below).

2.2. Immunohistochemical analysis

Rats for immunohistochemical analysis were terminally anaesthetized with sodium pentobarbitone (150 mg/kg, i.p., 17.00h) and perfused via the ascending aorta with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 0.1M phosphate buffer (PFA). Dissected brains were then post-fixed in PFA overnight at 4˚C and cryoprotected in 20% sucrose in 0.1M phosphate buffer at 4˚C overnight.

Sections of brain (12µm) were cut in the transverse plane (Leica CM1900 Cryostat; Leica Imaging Solutions Ltd, Cambridge, UK) and mounted on glass slides (SuperFrost Plus, VWR International, Poole, Dorset, UK). Sections were collected from across the SCN region (from approximately Bregma -0.3mm to -1.3mm, according to Paxinos & Watson (2005) for the rat, and an equivalent region for the mouse). Slides were dried briefly, and stored at -80 ºC prior to immuno-histochemistry. Proteins were detected by fluorescence immunohistochemistry using standard procedures established in our laboratory (Geoghegan & Carter, 2008; Wells et al, 2011); double immunohistochemical procedures involved combinations of two primary and two secondary antisera and were conducted only after individual single immunohistochemical procedures had been shown to exhibit cell-specific detection. The following primary antisera were used: (rabbit) anti-EGR1 (1/250; 15F7, Cell Signalling Technology, Beverly, MA, USA), (rabbit) anti-GFAP (1/400; G560AA, Promega, Madison, WI, USA), (mouse) anti-MeCP2 (1/200; 4B4, Active Motif, Carlsbad, CA, USA), (mouse) anti-NeuN/Fox-3 (1/100; MAB377, Millipore, Temecula, CA, USA), (rabbit) anti-SOX2 (1/1000; 39823, Active Motif), (mouse) anti-SOX2 (1/100; L1D6A2, Cell Signalling Technology). Specificity of the EGR1, GFAP, NeuN and (mouse) SOX2 antisera has been verified in previous publications (Man et al, 2007; Wells et al, 2011). Specificity of the (rabbit) SOX2 antisera was verified initially by demonstrating similar SOX2 detection compared with the mouse SOX2 antibody and, in addition, we have conducted an immunoblot analysis to verify detected protein mass and specificity (see Results). In this study, we used NeuN and

MeCP2 co-localization to confirm a neuronal identity for particular populations of SOX2+ cells; other populations of NeuN/MeCP2-negative cells (see Results) are simply assumed non-neuronal unless verified as such by GFAP co-localization.

The primary antisera listed above were used in combination with appropriate species-specific, fluorophore-tagged, secondary antisera (diluted 1/500): Cy3-conjugated sheep anti-rabbit IgG (Sigma-Aldrich UK, Bournemouth, UK) Cy3-conjugated donkey anti-mouse IgG (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA), Alexa Fluor 488-conjugated donkey anti-mouse IgG (Life Technlogies, Paisley, UK). Prior to microscopic examination, brain sections were sealed under coverslips using Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA). Sections were viewed with a fluorescence microscope (Leica DM-LB). Images were captured using a Leica DFC-300FX digital camera and Leica QWin software (V3), and montaged in Photoshop (CS2, Adobe Systems Inc., San Jose, CA, USA). Quantification of cell numbers was performed on images obtained from the light pulse experiment (approximately -0.5mm from Bregma [Paxinos & Watson, 2005]; x200 magnification; SOX2 & DAPI immunofluorescence merged). Using ImageJ (http://imagej.nih.gov/ij/), a grid was placed over the SCN area, and cells (total, and SOX2+ cells within the SCN boundary) were counted manually using 'Cell Counter' (http://imagej.nih.gov/ij/plugins/cell-counter.html) to mark counted cells. A total of 12 SCN were sampled (2 SCN from each of three rats in each of the two conditions (control and light pulse). The total number of cells counted was 3756 (Mean number per SCN, 313.0±11.0, n=12). To compare SOX2 expression between the light pulse and control conditions, SOX2+ cells were expressed as a percentage of total cells in each SCN, and the two percentage values for each rat were meaned to generate two sets of values for statistical comparison (n=3 control rats vs. n=3 light pulse rats). Statistical comparison of these experimental groups was conducted

using Student t test for independent samples (significance level $p<0.05$; SPSS 20, SPSS Inc., Chicago, ILL, USA). The same counting software was also used to estimate SOX2+ cell numbers in the piriform cortex (see Results).

2.3. Immmunoblot analysis

In order to provide additional validation for the (rabbit) SOX2 antibody, an immunoblot analysis was conducted using standard procedures established in our laboratory (Wells et al, 2011). A male rat (PN50) was humanely killed at 12.00h and the hippocampus was rapidly dissected. An extraction procedure was then used to generate hippocampal protein extracts that have relative enrichment for cytoplasmic and nuclear proteins as described and validated previously (Humphries et al, 2004). Western (immunoblot) analysis was conducted using standard procedures established in our laboratory (Wells et al, 2011) and a chemiluminescence detection system (ECL plus, GE Healthcare, Little Chalfont, Bucks., UK).

3. RESULTS

Immunohistochemical analysis of the adult rat brain revealed abundant SOX2 protein expression in particular areas of the hypothalamus (Fig.1A). SOX2 expression was abundant both within the SCN (approximately 70% of SCN cells were SOX2+, see below) and also in areas immediately dorsal to the SCN including the periventricular nucleus (PeN; Figs. 1A-D; see below) and ventricular ependymal cells (Ferri et al, 2004). Lateral to this hypothalamic area (see Fig. 1A) and in other hypothalamic nuclei such as the supraoptic nucleus (SON; not shown), the abundance of SOX2+ cells decreased markedly. SOX2+ cells were also generally scarce across the rest of the brain examined in this study (see exceptions below). Authenticity of the SOX2

immunoreactivity detected in the rat SCN was verified, firstly by immunoblot analysis using the rabbit anti-SOX2 antibody that detected a single protein band of the anticipated size (Fig. 2A), and secondly by confirming immunohistochemical detection in the SCN with a different SOX2 antibody (Fig. 2B). We also showed that the pattern of SCN SOX2 expression was retained in older (PN293) adult male rats (Fig. 2C). In another control, we showed that omission of the primary antibody from the procedure resulted in a complete loss of 'SOX2' detection (Fig. 2D&E). The described pattern of hypothalamic SOX2 expression was also shown to be conserved in the mouse (Fig. 2D); this mouse protein data is supported by Allen Brain Atlas *in situ* hybridization data (http/mouse.brain-map.org/; Experiment: 77280331) indicating that *Sox2* mRNA is similarly enriched in the SCN/dorsal SCN region. Finally, in additional positive controls, we showed that the SOX2 antibodies also detected well-characterized SOX2+ progenitor cell populations in both the sub-granular zone (SGZ) of the hippocampus (Fig. 2G) and sub-ventricular zone (SVZ, Fig. 2H).

The pattern of SOX2-immunoreactive cells in the SCN area of the rat brain indicated that these were neurons, given the abundance of SOX2+ cells within the SCN and the relative scarcity of expression in the white matter of the optic chiasm, for example (see Fig.1). To confirm neuronal expression of SOX2 in the SCN, we used a well-established neuronal marker antibody for NeuN (Kim et al, 2009; Mullen et al, 1992) in co-localization studies (Fig.3A-C). Although NeuN is (unusually) expressed in only a (ventral) sub-set of SCN neurons (Geoghegan & Carter, 2008), we demonstrated that SOX2 is clearly co-localized in some but not all of the SCN NeuN+ neurons. In order to confirm that SOX2 is neuronal in the dorsal SCN, we used another neuronal marker antibody for MeCP2 (Kishi & Macklis, 2004) that is expressed across SCN neurons (Alvarez-Saavedra et al, 2010). This analysis revealed that SOX2 is

expressed in a majority of MeCP+ SCN neurons (Figs. 3D-F) although a number of SOX2+/MeCP2-negative cells indicated the presence of a separate, smaller population of SOX2+ non-neuronal cells in the SCN. We also observed that the relative level of MeCP2 immunoreactivity across SCN neurons was similar to that in extra-SCN neurons (Fig.3G) indicating that immature phenotypes in these neurons (Geoghegan & Carter, 2008) do not include low MeCP2 expression.

In contrast to the SCN, where SOX2 is abundantly expressed in neurons, we found no evidence of SOX2 in extra-hypothalamic neurons (Figs. 3H-J). As noted above, we verified the presence of SOX2+ progenitor cells in the SGZ and SVZ (Figs. 2G&H), and also identified rare NeuN-negative/SOX2+ cells in the piriform (Fig. 3H) and other (Fig. 3I) cortical regions. The relative scarcity of SOX2+ cells in the cortex relative to the abundant expression in the SCN region is illustrated with reference to Fig. 3H; SOX2+ cells are estimated at 3.8% of total DAPI+ cells in this image. The probable identity of cortical cells as a rare astroglial/ progenitor population identified previously in the rat cortex (Komitova & Eriksson, 2004) was partly confirmed in the present study by co-localization with GFAP (Fig. 3I). An additional novel observation of the present study is that we also identified another brain area with abundant SOX2 expression, namely the circumventricular subfornical organ (SFO, Fig. 3J). However, in the SFO, all SOX2 cells were found to be NeuN-negative and therefore likely to be a population of non-neuronal cells. Other circumventricular organs were not evaluated.

The surprising finding of abundant neuronal expression of SOX2 in the adult rat and mouse SCN raises questions about the functional integrity of these neurons given the presence of a developmental protein. In order to verify that SOX2+ SCN neurons

are established within functional SCN neuronal networks, we used a physiological paradigm to investigate neuronal activation. In response to a light stimulus (see Man et al, 2007), the immediate early gene protein EGR1 is induced in ventral SCN neurons (Fig. 4A-D). In co-localization studies, we showed that SOX2 protein is highly expressed in a sub-set of these light stimulus-activated neurons (Fig. 4E&F). There was no indication of gross changes in the expression of SCN SOX2 protein following the light stimulus (Figs. 4G&H); values for SOX2+ cells expressed as a percentage of total SCN cells at one rostro-caudal level were found to be very similar in parallel-processed groups of control and light-stimulated rats (Figs. 4I&J).

In addition to the novel finding of SOX2 in adult SCN neurons, we also observed abundant SOX2+ cells in the region of the hypothalamus dorsal to the SCN (Fig. 1A-D) that includes the hypothalamic periventricular nucleus (PeN). In order to identify the nature of this additional hypothalamic population, we performed further co-localization studies with neuronal markers (Fig. 5A-F). This analysis showed that NeuN+/MeCP+ neurons of the PeN often expressed SOX2 although additional populations of both (NeuN+MeCP+)/SOX2-negative neurons and SOX2+/(NeuN-negative&MeCP-negative) (presumed) non-neuronal cells were also evident (Fig. 5A-F).

4. DISCUSSION

Sox2 is a fundamental neural developmental factor, generally considered restricted to developing neurons and neural progenitors, that acts, partly, to maintain cells in an undifferentiated state (Graham et al, 2003; Sarkar and Hochedlinger, 2013). Our novel finding of abundant SOX2 in rat hypothalamic cells (identified as mature neurons by NeuN/MeCP2 expression) until at least 9 months of age in rats is therefore evidence of hypothalamic neoteny. This finding accords with our previous

work on neuronal immaturity in the rat hypothalamus in which we demonstrated both adult expression of the developmental protein DCX in a specific population of SCN neurons and also a remarkable absence of the mature neuronal marker protein NeuN from the majority of SCN neurons (Geoghegan & Carter, 2008). Similar immature phenotypes have been confirmed in more recent studies (Morin et al, 2011; Saaltink et al, 2012), although the presence of species variation in specific developmental molecule and cellular distribution indicates that these phenotypes may potentially be downstream of a common regulatory mechanism. Our demonstration of SOX2 protein expression across the SCN of both adult rats and mice has now identified one candidate regulator.

Previous work on *Sox2* expression in the adult rodent brain has principally identified populations of progenitor/stem cells that require SOX2 for multipotency (Arnold et al, 2011; Brazel et al, 2005; Komitova and Eriksson, 2004). In the present study, we have confirmed these findings with respect to defined populations of SOX2+ progenitor cells in the SVZ and SGZ and the functional role of these populations is well established (Kempermann, 2012). There have also been some reports of *Sox2* expression in differentiated neurons (Ferri et al, 2004; Kang and Hebert, 2012) although these have been limited to scattered cells in cortical and other extra-hypothalamic regions of the brain. In one study, conditional knockout of *Sox2* in the adult mouse resulted in some loss of brain function and neurodegeneration that was attributed to a potential role for *Sox2* in the maintenance of some populations of adult neurons (Ferri et al, 2004). Our analyses of adult male Sprague-Dawley rats and C57BL/6J mice has not revealed significant populations of SOX2+/NeuN+ neurons in extra-hypothalamic areas at the rostro-caudal levels examined in the present study. Indeed, another novel site with large numbers of SOX2+ cells identified here, the SFO, exhibited an absolute separation of SOX2+ and NeuN+ cells, indicating that the

large population of SOX2+ cells in this circumventricular site are likely to be progenitor/glial cells. Further studies are required to investigate both the functional relevance of the SFO SOX2+ cell population and the requirement for such a concentration of SOX2+ cells in this site.

With regard to the role of SOX2 in the adult hypothalamus, particularly the SCN, it is clear from our current work that SOX2 is present in mature functional neurons that are able to participate in physiological responses, for example to light stimulation. This indicates that SOX2+ cells in the SCN do not form a pool of non-functional 'reserve' neurons that are independent of established neuronal networks underlying SCN regulation of circadian rhythms (Reppert & Weaver, 2002). However, in the light of recent important studies (Spitzer, 2012), it is conceivable that SOX2 (and other associated proteins) may confer a neuronal re-specification functionality that could permit neurotransmitter switching in response to adaptive demand (Dulcis et al, 2013). However, a requirement for this type of functional capacity in a majority of neurons across the SCN (and PeN) is not clear because a general re-specification capability would appear incompatible with the need for mature neuronal network integrity. Our finding that SOX2 is expressed in neurons across the SCN indicates that the functional role is general rather than specific; the rodent SCN has a complex anatomy with many neurochemically and functionally distinct groups of neurons organized into SCN sub-divisions (Moore et al, 2002). Therefore, based on levels of neuronal SOX2 alone (see below), it appears unlikely that this factor is functionally associated with one particular aspect of SCN physiology. Previously (Geoghegan & Carter, 2008), we have speculated that immature neuronal phenotypes in the SCN may underlie a general requirement for enhanced neuronal plasticity during the marked daily fluctuations in activity in this nucleus; this may be limited to synaptic 'adjustments' (Girardet et al, 2010) rather than gross changes in cellular phenotype.

The adult hypothalamic SOX2 identified in the current study may also relate to an identified involvement in regulation of the gonadotrophin axis (Jayakody et al, 2012). Further studies are required to investigate potential *Sox2*-interacting factors in these hypothalamic nuclei; SOX2 often functions by antagonizing the activity of other factors (Sarkar & Hochedlinger, 2013) and clearly the overall balance of transcriptional regulators, rather than the presence of a single factor, is the critical determinant of functional outcome in these neurons. A recent report of *Sox3* expression in the adult mouse hypothalamus (Rogers et al, 2013), reflecting parallel expression/roles of *Sox2* and *Sox3* in development (Sarkar & Hochedlinger, 2013), indicates one potential co-factor.

In conclusion, we have identified novel populations of SOX2+ cells in the adult rat brain, both a neuronal population in the SCN and presumed (NeuN-negative) non-neuronal cells in the SFO. The abundance of cells in both of these sites indicates a general functional relevance that requires further investigation. The finding of an additional immature characteristic of SCN neurons substantiates our previous observations (Geoghegan & Carter, 2008) and provides a basis for identifying molecular mechanisms that permit these characteristics in mature neurons. With respect to Sox2 gene regulation, an analysis of cell-specific gene regulation in the SCN may provide important insights into the role of particular neural *Sox2* enhancers first identified by Uchikawa et al (2003). Our findings challenge established assumptions about the development of neuronal identity that is generally considered to involve the establishment of a pre-specification ‘ground state’ during which the expression of developmental factors such as *Sox2* are rapidly terminated (Fishell & Heintz, 2013). Further analysis of the SOX2+ neurons identified here may also provide valuable insights into the mechanisms underlying this process.

5. ACKNOWLEGEMENTS

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7. FIGURE LEGENDS

Fig.1. Widespread expression of SOX2 protein immunoreactivity in the adult rat hypothalamus. Representative fluorescence microscopic images of P50 brain showing immunohistochemically detected SOX2+ cells. **A**. SOX2 is expressed extensively in the SCN at the base of the 3V and also in areas dorsal to the SCN, including the PeN, adjacent to the 3V. Note that the number of SOX2+ cells is relatively lower in both the adjacent anterior hypothalamus (area to the right of the dashed line) and also in the OC. Scale bar = 100µm. **B-D**. Detail of (A) showing the distribution of SOX2 across a single SCN and localization in DAPI+ cells. Scale bars = 50µm. Abbreviations: 3V, third ventricle; OC, optic chiasm; PeN, periventricular nucleus; SCN, suprachiasmatic nucleus.

Fig.2. Conservation of authenticated hypothalamic SOX2 expression in old adult rats and mice. **A**. Immunoblot analysis using the rabbit anti-SOX2 antibody to demonstrate specific detection of an approximately 34kDa protein band that is enriched in nuclear (N) compared with cytoplasmic (C) brain hippocampus protein extracts (25µg/lane). Note that SOX2 protein in the hippocampal extracts reflects the abundant (non-neuronal) expression of SOX2 in the subgranular zone of the dentate gyrus. **B&C**. Representative fluorescence microscopic images of P50 (B) and P293 (C) rat brain showing immunohistochemically detected SOX2+ cells that are abundant in the SCN. The image in (B) was obtained with the mouse anti-SOX2 antibody (L1D6A2, see Methods). This was used to verify SOX2 localization obtained with the rabbit antibody (used for all other images). Scale bars = 50µm. **D&E.** Representative fluorescence microscopic images of P50 rat brain showing sections processed in parallel for immunohistochemical detection of Sox2 where the primary antibody was omitted in E. Scale bars = 100µm. **F**. Representative fluorescence

microscopic image of mouse brain showing a similar pattern of SOX2 expression in the mouse hypothalamus. Scale bar = 100µm. Abbreviations as Fig.1. CP, choroid plexus; GCL, granule cell layer; LV, lateral ventricle; SGZ, subgranular zone of the dentate gyrus; SVZ, sub-ventricular zone.

Fig.3. Adult rat brain SOX2 expression is largely neuronal in the SCN but is non-neuronal in many other brain regions. Representative fluorescence microscopic images of rat brain showing immunohistochemically detected SOX2+ cells. **A-F**. SOX2 in NeuN+ and MeCP+ neurons of the SCN. Note that NeuN+ neurons are restricted to the ventral SCN in rats (see text). Arrows show clear examples of SOX2 co-localization with the neuronal marker proteins. Scale bars = 20µm. G. Representative fluorescence microscopic image of rat brain showing immuno-histochemically detected MeCP2 across the SCN. Note that similar levels of MeCP2–associated fluorescence are observed in both SCN and extra-SCN neurons. Scale bar = 50µm. **H**. SOX2+ cells in the piriform cortex that are distinct from NeuN+ neurons. Arrows in H indicate the rarity of SOX2+ cells in the piriform region. Scale bar = 50µM. **I.** Co-localization of SOX2 and GFAP in a single cell of the somatosensory cortex of the rat. Scale bar = 20µm. **J**. Abundant SOX2+ cells in the subfornical organ that are uniformly NeuN-negative. Scale bar = 20µM. Abbreviations as Fig.1; Piri, piriform cortex; SFO, subfornical organ.

Fig.4. SOX2 is expressed in light-responsive neurons of the adult rat ventral SCN. Representative fluorescence microscopic images of rat brain showing immunohistochemically detected SOX2+ cells. **A-D**. EGR1 protein is induced in the ventral SCN by a light stimulus (see text). Scale bars = 50µm. **E&F**. SOX2 is expressed in a sub-set of the light-induced EGR1+ neurons. Arrows in the image

detail (F) show clear examples of SOX2/EGR1 co-localization. Scale bars = 50µm (E), 20µM (F). **G&H.** The abundance and distribution of SOX2 protein is similar in control (G) and light-stimulated (H) rats. Scale bars = 50µm. **I.** Illustration of a grid and SCN outline used for the estimation of SOX2+ cells as a percentage of total (DAPI+) cells in merged images of the SCN. Scale bar = 50µm. **J.** Histogram showing the relative percentages of SOX2+ cells in the SCN of control (CON, n=3 rats) and light-stimulated rats (LIGHT, n=3 rats). Values are Mean ± S.E of SOX2+ cells expressed as a percentage of total SCN cells (CON vs. LIGHT; p=0.487, Student's t test). Abbreviations as Fig.1

Fig.5. SOX2 is expressed in NeuN+/MeCP+ neurons of the hypothalamic periventricular nucleus. Representative fluorescence microscopic images of rat brain showing immunohistochemically detected SOX2+ cells in the periventricular nucleus. **A-C**. Series of images showing NeuN co-localization. **D-F**. Series of images showing MeCP2 co-localization. Small vertical arrows show abundant SOX2-co-localized cells large horizontal arrows show SOX2+/(NeuN/MeCP2-negative) cells, and large vertical arrows show (NeuN+/MeCP2)/SOX2-negative neurons. Scale bars = 20µm.