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Sensory neurons in the spinal cord of nominal female embryos in the marine turtle *Lepidochelys olivacea* respond to shifts in incubation temperature: implications for temperature dependent sex determination

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ABSTRACT

Gonadal determination in marine turtles depends on incubation temperature. The mechanisms that spark off this process remain unclear. Previously, we proposed that sensory nerves reaching the gonadal *primordium* in nominal female embryos of *Lepidochelys* (*L*) *olivacea* may sense and signal incubation temperature. These nerves could later trigger ovarian determination by releasing neurotransmitters in a code constructed based on the thermal information (Gutierrez-Ospina *et al.*, Acetylcholinesterase-positive innervation is present at the undifferentiated stages of the sea turtle *Lepidochelys olivacea* embryo gonads: implications for temperature-dependent sex determination, J. Comp. Neurol. 410 (1999) 90-98). The hypothesis briefly described, however, has been recently refuted under weak theoretical grounds and experimental misinterpretations (see introduction). Here, we present preliminary results that show that nominal female embryos have sensory neurons located in the dorsal horn laminae I and II of the lumbar spinal cord that display increased c-Fos-like immuno-staining after being incubated either at 15°C or 50°C. Because these spinal neurons are the primary central target of dorsal root ganglion neurons that innervate the urogential crest, these observations keep open the possibility that gonadal sensory nerves indeed signal thermal information that could later be used to trigger or instruct ovarian specification in marine turtles.

Keywords: Reptiles; Ovarian Determination; c-Fos; Incubation Temperature; Sensory Neurons

1. INTRODUCTION

Sex determination in marine turtles depends on incubation temperature [1]. In general, low (26-27°C) or high (32-33°C) incubation temperatures give rise to males or females, respectively [2,3]. Sexual differentiation may be re-directed if eggs are switched from one incubation temperature to the other during the thermo-sensitive period of sex determination [3]. As this period progresses, the effect of shifting the eggs between incubation temperatures on sex determination wanes until fully disappearing [3]. The mechanisms underlying temperature dependent sex determination (TSD) in reptilian species have been the subject of intense research and debate over the past several decades. As a result, it is now widely accepted that incubation temperature channels gonadal, and likely other organs [4], determination and/or differentiation in part by regulating the activity of

steroidogenic enzymes and the local production of sexual steroids [1,5-7]. Also, recent evidence supports that incubation temperature may modulate the expression of sex determining genes in vertebrate species that display TSD [8-11]. In spite of the progress achieved, the mechanism by which thermal information is translated and funneled towards the activation of the genomic-biochemical pathways that ultimately lead to TSD remains elusive (for a critical review see [12].

Three, likely complementary, hypotheses have been put forward to explaining how TSD might be triggered. In one of these scenarios, it is assumed that there are temperature-sensitive promoters that induce or repress the expression of gonadal genes whose products are key regulators of the gene cascades and/or biochemical pathways that determine gonadal fate [1]. Unfortunately, no empirical evidence has been published so far supporting this notion [12]. The second hypothesis elaborates on the existence of gonadal biochemical pathways that are rendered sensitive to incubation temperature by means of metabolic intermediaries (e.g., CO_2) whose cellular concentrations shift depending on the incubation temperature [4]. Although some experimental evidence supports this notion, the fact that the synthesis, degradation and/or the activity of fundamental enzymes may shift rapidly as incubation temperature does it in embryos of poikilothermic species [13-15], suggests that this kind of responses may not be tightly controlled by specific sex-determining molecular cascades [16,17].

The third hypothetical scenario calls for the participation of the nervous system. In this model, temperature responsive sensory nerve fibers located within the gonads could signal thermal information and locally release neurotransmitters after being stimulated [18]; sensory nerves are long known to display efferent functions [19,20]. The idea just described has been recently refuted [6-8] based on the observation that isolated cultured *L. olivacea* gonads either maintain or down regulate sox-9 expression when incubated at masculinizing or feminizing temperatures, respectively (although see [21] for conflicting results). We believe, nonetheless, that this conclusion is undermined by observations showing that in some vertebrates 1) nerve fibers and terminals are closely associated with embryonic, ovarian estrogen-producing interstitial cells [22-25]; 2) cultured embryonic gonads retain this innervation for several days after being severed from the central nervous system [24,25]; 3) synaptic terminals remain functional and responsive for several days *in vitro* after being separated from their neuronal origin specially in turtles [26-28]; 4) neurotransmitter release in isolated terminals is sensitive to temperature [29,30]; and 5) aromatase gene transcription and enzymatic activity may be regulated by catecholaminergic, dopaminergic and glutamatergic inputs [31,32].

Hence, in this work we intended to re-open the possibility that gonadal sensory innervation signals thermal information by showing neuronal activation in the dorsal horn of the lumbar spinal cord following thermal stimulation in *L. olivacea* nominal female embryos before ovarian specification takes place. Nominal female embryos were used because sensory innervation is readily established between the lumbar spinal cord and the urogential before ovarian determination is triggered [18].

2. MATERIAL AND METHODS

2.1. Animals and Tissue Sampling

The experiments were performed using eggs collected in La Escobilla beach (96°27’16’’W, 15°40’36’’N), Oaxaca, México. The eggs were transferred to the laboratory in vermiculite-made artificial nests. Once in the laboratory, a total of 16 eggs were placed in an oven at 33°C until the embryos reached the stage 23-24 of development; three embryos taken randomly were used to corroborate the developmental stage based on external morphological features [3]. Then, a group of the eggs was switched from 33°C to 15°C (n = 5). Other group was changed from 33°C to 50°C (n = 5) during half an hour and then returned to 33°C for 30 minutes. This physiologically meaningful temperature range was defined based upon the discrimination features, the physiological responses and the psychophysical properties of warm and cold receptors in mammals [33], and considering the range of incubation temperature [34] and of the ocean water in which turtles normally navigate [35]. Also, experiments conducted in avian eggs have revealed that one hour of incubation in a temperature different from the standard base line incubation temperature is sufficient to modify various embryonic metabolic parameters [36]. By the end of the 30 minutes re-acclimation period, the embryos of both groups were rapidly dissected and fixed in buffered paraformaldehyde (4%) at 4°C overnight. Control eggs (n = 3) were kept at 33°C throughout the experiment and their embryos were processed as described before. The following day, the embryos were transferred to a solution containing sucrose (20%) at 4°C until they sank two days later. The embryos were then embedded in OCT compound, frozen in 2-methyl butane pre-chilled with dry ice and cut (10 µm) entirely in a coronal plane using a cryostat. One section every 100µm was sampled and mounted onto gelatin coated slides and process through immunofluorescence (see below). In addition, a batch of hatchlings (n = 3) was used to collect brain samples for testing the specificity of the antibody through western blot analyses.

2.2. Western Blot and Antibody Specificity

L. olivacea hatchlings (10 days old) were euthanized with pentobarbital (45mg / Kg of body weight) and decapitated. The forebrain was rapidly dissected and placed into microcentrifuge tube containing RIPA buffer (25 mM Tris•HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, pH 7.6), supplemented with the complete protease inhibitor cocktail used according to the manufacturer's instructions (Roche Applied Science). The samples were homogenized by sonication (40W) and centrifuged at 12,000 revolutions per minute for 20 minutes at 4°C. The supernatants were collected and the protein content was estimated by using the bicinchoninic acid protein assay (Pierce) read at 560 nanometers. An aliquote of 75 µ icrograms of protein per sample was electrophoresed through one dimension SDS-polycacrilamide gels (12.5%) at 150 volts during 90 minutes at 4°C. Proteins were transferred to nitrocellulose membranes using a semi-dry system (BioRad) during 50 minutes at 0.3A of constant current. The quality of the protein transference was evaluated by using Ponceau's staining. After destaining, nitrocellulose membranes were incubated with blocking solution (5% non-fat milk, 3% goat serum, 0.1% Tween-20 in TBS). The membrane was then incubated with rabbit anti-human c-Fos polyclonal primary antibodies (Santa Cruz sc-52) overnight at room temperature (1:200 in blocking solution). This antibody recognizes a highly conserved amino-terminal sequence of c-Fos (residues 4-17) that appear to be present in turtles and amphibians [37,38]. After a gently wash in TBS added with 0.1% Tween-20, the membranes were incubated with the corresponding biotin-conjugated secondary antibody (AP187B, Chemicon) during 2 hours (1:800 in blocking solution) at room temperature. Then, the membranes were washed and incubated with avidin-peroxidase for 1 hour at room temperature following the supplier's recommendations (Vector Laboratories). Peroxidase activity was revealed using a chemo-luminescent substrate according to the manufacturer's guidelines (Immobilon Western, Millipore) and documentation was performed using the Gel Logic 1500 system (Kodak Molecular Imaging).

2.3. Immunofluorescence

Tissue sections were incubated with blocking serum containing bovine albumin (1%) and triton X-100 (0.3%) in phosphate buffer (0.1M, pH 7.4) for 3 hours at room temperature. After three 15 minute washes, the sections were incubated with primary antibodies raised against c-Fos diluted 1:1000 in blocking serum at 4°C for 12 hours. After three washes, the sections were incubated with a secondary antibody goat anti-rabbit IgG coupled to fluorescein diluted 1:200 in blocking serum for three hours at room temperature. The incubation with the primary antibody was omitted in control experiments to rule out false positive results. Following the last washing step, sections were coverslipped with anti-fading mounting medium (Dako). Slides were then observed in an Optiphot Nikon epifluorescence microscope and digital images were taken using a Nikon coolpix digital camera. Figures showing immunocytochemical and western blot results were elaborated by using Adobe Photoshop CS2 (version 9.0.2).

3. RESULTS

3.1. Antibody Specificity

Figure 1 illustrates a representative Western blot showing the single protein band that was immuno-reactive for c-Fos in samples of the *L. olivacea* hatchlings forebrain. Such a band displayed an approximate molecular weight of 40kDa, a weight that is similar to that published for one of the c-Fos nuclear isoforms reported in the frog *Rana esculenta* [38].

3.2. Spinal Cord c-Fos Immunoreactivity

Once established the specificity of the antibodies based on molecular weight equivalence, we evaluated whether the intensity and location of c-Fos-like immuno-staining shifted in neurons located in the dorsal horn of the embryonic lumbar spinal cord at the ontogenetic stage 23-24, before ovarian specification occurs [3]; these spinal

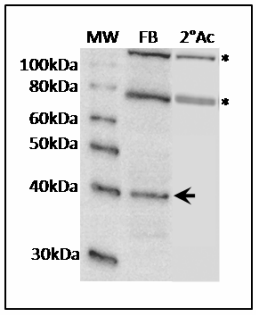

Figure 1. Digital image of a representative western blot stained for c-Fos obtained after running samples obtained from the *L. olivacea* hatchlings forebrain. Lane 1: MW-Molecular Weight Delete the dash in molecular markers; Lane 2: FB-Forebrain samples; Lane 3: Background staining associated with the secondary antibody (asterisks; 2° AC). The arrow indicates where the c-Fos immunoreactive band is identified.

neurons are the primary central target of dorsal root ganglion neurons that innervate the urogential crest [18]. Two patterns of cellular staining were consistently observed. Embryos exposed to 15°C had neurons exhibiting cytoplasmic staining (**Figure 2(a)**). In contrast, when embryos were exposed to 50°C, the majority of neurons displayed nuclear staining (**Figure 2(b)**). In our hands, embryos kept at their original incubation temperature did not show appreciable expression of c-Fos in the spinal cord (**Figure 2(c)**). Omission of the primary antibody resulted in a negative c-Fos immunostaining (not shown). Finally, even though the pattern of cytological staining differed between embryos exposed to high or low incubation temperatures, the distribution and relative amount of immunopositive neurons was similar between groups; numerous c-Fos like immunopositive neurons essentially occupied laminae I and II of the dorsal horn at the lumbar segments of the spinal cord.

4. DISCUSSION

Sex determination in marine turtles depends on incuba tion temperature. Although the molecular and biochemical processes that channel ovarian and testicular determination during TSD are now better understood, the precise mechanisms by which it is triggered remain unclear. We have previously suggested that sensory nerves located inside the undifferentiated gonad of nominal *L. olivacea* female embryos might signal thermal information and, upon activation, might release neurotransmitters that could turn on the cascade of events leading to sex determination [18]. In this work, we provide evidence that strengthens this possibility by showing that sensory neurons located in the dorsal horn of the lumbar spinal cord respond to shifts in incubation temperature, as monitored by the increment of the staining intensity of a c-Fos-like protein. The spinal levels where c-Fos-like positive neurons were mapped precisely in sites that receive primary sensory afferents incoming from the urogenital crest [18]. Because sensory nerves exert efferent functions on their targets [19,20], we believe this primordial connectivity might be an important component of the machinery triggering ovarian differentiation, even in the absence of activation of upper neural structures involved in thermal information processing.

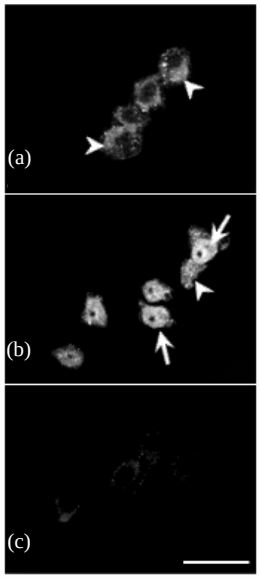

Figure 2. Digital photomicrographs showing examples of the cellular patterns observed for c-Fos staining in the dorsal horn of the spinal cord following the exposure of embryos at 15°C (a) or 50°C (b). Nuclear (arrows) and cytoplasmic (arrowheads) staining are indicated. (c) Illustrates c-Fos basal staining in the spinal cord of non-stimulated embryos. Scale Bar = 10 µm.

In spite of the implications that our results have on TSD conceptions, we must be prudent in interpreting the present data. Given the protocol used for eliciting neuronal activation, we cannot rule out that the increment in the intensity of c-Fos like immuno-staining observed in spinal cord sensory neurons might in part reflect heat stress-associated responses; this distinction may be crucial because c-Fos may exert pro-apoptotic actions [39]. However, in favor of our experimental design 1) it has been documented that increments of c-Fos availability can also counteract apoptosis and promote cell differentiation [40]; 2) Also increment in c-Fos availability and nuclear translocation facilitates cell proliferation [41]; 3) The thermal values used to stimulate the embryos were carefully selected based on what we know on the discrimination features, physiological properties and psychophysical responses of warm and cold receptors in mammals, the best characterized receptors in the animal kingdom [33]. We also considered the temperature range of the nests [e.g., 34] and of the ocean water in which marine turtles normally navigate [35]. Even so, we would concord that a definitive answer requires an unquestionable molecular identification of the neuronal phenotype that is involved specifically in thermal information processing in marine turtle embryonic spinal cord, as it has been shown for central neurons in *Caenorhabditis elegans* based on *LIM* homeobox gene expression [42].

An intriguing observation is related with the differential distribution of c-Fos inmunostaining in the cytoplasm or nuclear compartments of the activated spinal

cord neurons, depending on the temperature under which the embryos were incubated. Indeed, cytoplasmic staining was consistently observed in embryos incubated at 15°C, whereas nuclear staining was observed in those kept at 50°C. Although this result could be considered as irrelevant or even art factual, c-Fos-like cytoplasmic and/or nuclear staining following neuronal activation is not uncommon in phyla different from mammals [38, 41,43-47]. Indeed, it has been shown that c-Fos cytoplasmic staining reflects a reduced rate of translocation to the nucleus due to decreased metabolic rates [41,44], increased phospholipid metabolism [47] or diminished hormonal stimulation [38]. Further studies are necessary to explore each one of these possibilities.

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