The Journal of Experimental Biology 205, 3513–3518 (2002)
Printed in Great Britain © The Company of Biologists Limited
JEB4340

Discrete electrolytic lesion of the preoptic area prevents LPS-induced behavioral fever in toads

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Accepted 5 August 2002

Summary

The preoptic area (POA) plays an important role in fever in mammals, but the role of this region in fever in ectothermic vertebrates has never been assessed. Toads, like all ectotherms, regulate their body temperature (T_b) primarily by behavior and develop behavioral fever when injected with lipopolysaccharide (LPS). Therefore, we tested the hypothesis that the POA plays a role in the behavioral fever induced by LPS in the toad *Bufo paracnemis*. We made electrolytic lesions in the POA of toads (0.3 mA, 8 s) and measured preferred T_b using a thermal gradient. After a period of 24 h inside the gradient chamber, control, sham-operated and lesioned toads were systemically injected with LPS (200 μg kg^{-1}) or pyrogen-free saline. There was no significant effect of POA lesion in animals at their normal preferred T_b. LPS caused a significant increase in preferred T_b of control and sham-operated toads, but lesions in the POA abolished this response. These results indicate that the POA is an important site in the central nervous system of toads, and perhaps of all vertebrates, involved in the development of fever.

Key words: behavioral thermoregulation, amphibian, *Bufo paracnemis*, thermoregulatory set point.

Introduction

The preoptic area (POA) is a region at the junction of the telencephalon and diencephalon of the vertebrate brain that plays an important role in many functions, including reproductive behavior and thermoregulation (Boulant, 1998; Butler and Hodos, 1996). The role of the POA in thermoregulation has been most extensively studied in mammals. This region is thought to integrate information about local brain temperature and other body temperatures and to control the level of output for a set of thermoregulatory responses that are most appropriate for the given internal and environmental temperatures (Boulant, 1998). A few studies have indicated a role for the POA in the regulation of body temperature (T_b) of non-mammalian vertebrates such as fishes (Nelson and Prosser, 1979, 1981a,b), reptiles (Cabanac et al., 1967; Khromer and Crews, 1987) and birds (Nakashima et al., 1987; Necker and Gnuschke, 1989). For amphibians, no data exist about the role of the POA in thermoregulation.

Although evidence has shown that the POA is an important thermoregulatory site in some ectotherm species, the neural control of fever in these animals has received no attention. Fever is a regulated increase in T_b that is often described as a rise in the thermoregulatory set point (Kluger, 1991). In mammals, fever is produced by the coordinated actions of many central nervous system (CNS) regions as an adaptive response to infection. Some preoptic neurons not only sense changes in deep body temperature but are also affected by pyrogens that act on these neurons to cause a number of physiological and behavioral responses that elevate T_b (Boulant, 1998).

With few exceptions, both endothermic and ectothermic vertebrates (as well as invertebrates) develop fever in response to injections of exogenous pyrogens such as lipopolysaccharide (LPS; endotoxin), viruses, Gram-positive bacteria and yeast. LPS, which is the most purified form of a compound from the cell wall of Gram-negative bacteria, usually *Escherichia coli*, has been extensively used to induce fever in experimental animals (for a review, see Kluger, 1991). Recently, we demonstrated that the toad *Bufo paracnemis* develops fever after systemic injection of LPS (Bicego-Nahas et al., 2000), but the possible sites in the CNS involved in this response have not been assessed.

In the present study, we tested the hypothesis that the POA is important for the development of behavioral fever in *B. paracnemis*. To this end, we evaluated the effect of electrolytic lesions in the POA on preferred T_b and LPS-induced behavioral fever in *B. paracnemis*.

Materials and methods

Animals

Male toads (*Bufo paracnemis* Lutz) weighing 150–250 g were collected in the vicinity of Ribeirao Preto, Sao Paulo

state, Brazil during the rainy spring and summer, when the experiments were performed. The toads were maintained in containers with free access to water and basking area. All animals were fed cow liver twice a week up to 2 days before surgery, and the experiments were performed 4 days after surgery. Each animal was used only once and all experiments began at approximately 16:00 h. Animal care was carried out in compliance with guidelines set by COBEA (Colegio Brasileiro de Experimentaçao Animal).

Surgical methods

Animals were anesthetized by submergence in an aqueous 0.3% solution of 3-aminobenzoic acid ethyl ester (MS-222, Sigma, St Louis, MO, USA). The animal's head was then fixed to a David Kopf stereotaxic apparatus (Model 900 Small Animal Stereotaxic, Tujunga, CA, USA) and the skin covering the skull was removed with the aid of a bone scraper. An opening was made in the skull above the diencephalon region using a small drill (Model FM 3545, Foredom Electric, Bethel, CT, USA). A 0.4 mm tungsten electrode (A-M Systems, Everett, WA, USA) fixed to the electrode holder was positioned into the region of the POA (0.5 mm caudal to the telencephalon, 0.1 mm left and right from the midline, and 0.5 mm above the cranial base) according to the coordinates of the stereotaxic atlas for *B. paracnemis* (Hoffman, 1973). The electrode was connected to the anode of a source of continuous current (Ugo Basile, Comerio-VA, Italy, model 3500 Lesion Making Device) and the cathode was connected with a toothed clamp to the animal's paw, previously wrapped in cotton moistened with physiological saline. The bilateral lesions were made with a 0.3 mA current applied for a period of 8 s. After lesion, the orifice was filled with bone wax and acrylic cement. Sham-operated toads were similarly prepared, but no current was passed through the electrode. The experiments were performed 4 days after brain surgery. All animals looked perfectly healthy after experimentation, even the lesioned ones.

Measurement of preferred T_b

Preferred T_b was determined in a thermal gradient chamber (1.50 m long × 0.15 m high × 0.20 m wide) with an aluminum floor. One end of the floor was cooled to 10°C by a copper pad connected to a refrigerated water bath (VWR Scientific, 1160A, Niles, IL, USA). The other end was heated to 38°C by another copper pad connected to another water bath (Barnstead/Thermolyne, 310A, Dubuque, IA, USA). Petri dishes filled with tap water throughout the chamber provided access to water at all temperatures. An animal with a temperature probe, which was secured 2 cm into the cloaca with skin sutures, was placed in the center of the thermal gradient, and the thermistor output was continuously displayed on a chart recorder (Barnstead/Thermolyne, LR93125, Dubuque, IA, USA). Cold and warm water was used to calibrate the temperature probes before each experiment.

Experimental procedure

Experiments were performed on unanesthetized and unrestrained toads previously prepared as described. One toad was placed in the middle of the temperature gradient and left there for about 24 h. After this period, saline or LPS (from *Escherichia coli*, serotype 0111:B4, Sigma), was injected into the dorsal lymph sac of the animals, and T_b was monitored for 15 h after injections. The dose used, 200 μg LPS kg^{-1} body mass, was based on a previous study on toads (Bicego-Nahas et al., 2000). The gradient chamber was continuously flushed with humidified room air at a rate of 1.5 l min^{-1}.

Histology

At the end of each experiment the animals were anesthetized by submergence in 0.3% MS-222 and perfused through the heart with saline followed by 10% formalin solution. Immediately after, their heads were placed in 10% formalin for at least 2 days. The brains were then removed from the skull, histologically processed and immersed in paraffin, and serial coronal sections (17 μm) were cut and stained by the Nissl method for light microscopy determination of the electrolytic lesions.

Calculations and statistical analysis

Mean preferred T_b was determined every hour in all experiments from individual chart paper recordings by manual calculation based on a previous calibration. Two-way analysis of variance (ANOVA) was performed followed by Tukey–Kramer multiple comparisons test. All values are reported as means ± S.E.M. Values of $P<0.05$ were considered to be significant.

Results

Electrolytic lesion of the POA of toads

Fig. 1 shows electrolytic lesions placed in the POA of seven LPS-treated (Fig. 1B) and five saline-treated toads (Fig. 1C). Two different coronal sections (1 and 2, Fig. 1A) through the diencephalon are shown because they represent the most extensive site of lesion in the POA of each tested toad.

Effects of electrolytic lesion of the POA on preferred T_b *of toads in euthermic condition*

Toads selected a T_b of approximately 26°C, and no significant difference was observed among control, sham-operated and lesioned groups during a period of 24 h (Fig. 2).

Effects of electrolytic lesions of the POA on behavioral fever of toads induced by LPS

LPS caused a significant increase in preferred T_b ($P<0.05$) of control and sham-operated toads, a response that was abolished by electrolytic lesion of the POA. Lesions outside the POA did not alter the LPS-induced fever of toads (Fig. 3).

Discussion

The present study provides evidence that the POA plays a key role in the behavioral fever induced by LPS in toads, as

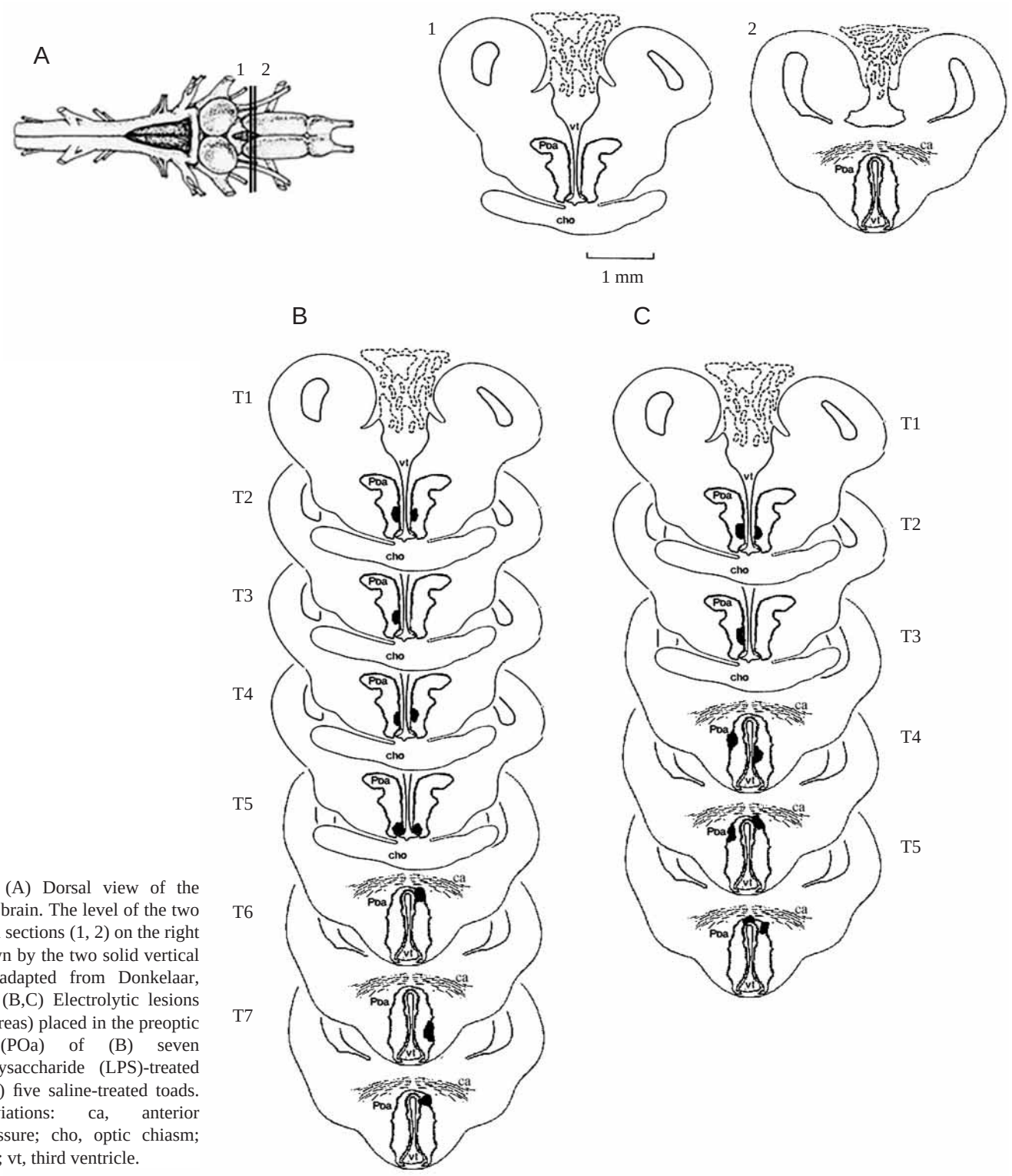

Fig. 1. (A) Dorsal view of the anuran brain. The level of the two coronal sections (1, 2) on the right is shown by the two solid vertical lines (adapted from Donkelaar, 1998). (B,C) Electrolytic lesions (dark areas) placed in the preoptic area (POa) of (B) seven lipopolysaccharide (LPS)-treated and (C) five saline-treated toads. Abbreviations: ca, anterior commissure; cho, optic chiasm; T, toad; vt, third ventricle.

electrolytic lesions in this region abolished this response in *B. paracnemis*. To our knowledge, this is the first study to report a role of the POA in behavioral fever in an ectotherm species. Ectotherms have been suggested to be an interesting model for the study of thermoregulation because they rely essentially on behavioral mechanisms for T_b control, which seem to be related to changes in the thermoregulatory set point (Heller et al., 1978; Crawshaw, 1980; Branco and Malvin, 1996; Branco and Steiner, 1999; Cabanac, 1998; Vaughn et al., 1974).

Role of the POA in thermoregulation

In mammals, the POA is considered to be the thermointegrative and thermosensitive site of the CNS, containing warm-sensitive and temperature-insensitive

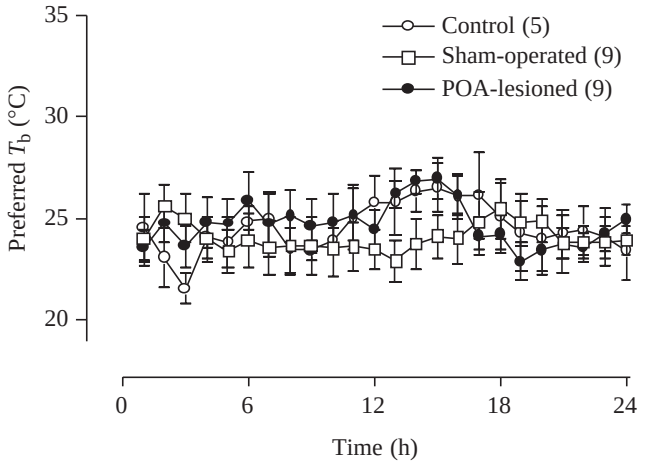

Fig. 2. Change in preferred body temperature (T_b) over time of control, sham-operated and preoptic area (POA)-lesioned toads during a period of 24 h. Values are means ± S.E.M. Numbers in parentheses indicate the number of animals.

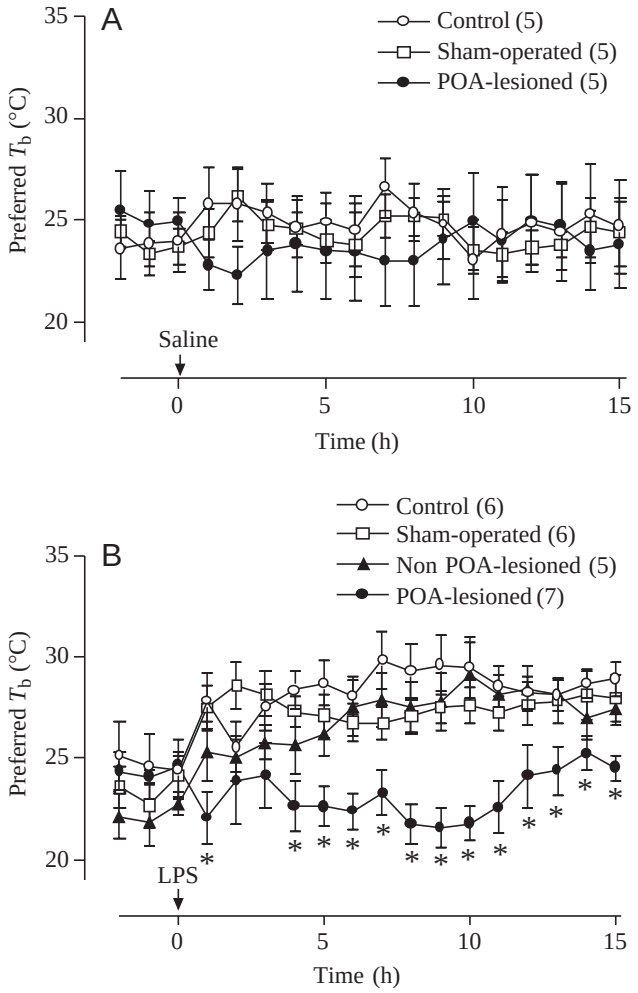

Fig. 3. Change in preferred body temperature (T_b) over time of control, sham-operated and preoptic area (POA)-lesioned toads injected into the lymph sac at time zero with either (A) pyrogen-free saline or (B) lipopolysaccharide (LPS) at a dose of 200 μg kg^{-1} body mass. A non POA-lesioned group injected with LPS is also plotted. Arrows indicate the time of injection. Values are means ± S.E.M. The asterisk indicates significant difference (P<0.05) from the control group at the same time points. Numbers in parentheses indicate the number of animals.

neurons, a balance of which determines the thermoregulatory set point (Boulant and Dean, 1986; Boulant, 1998, 2000). In the tested species, preoptic warming elicits heat-loss responses, including panting, sweating and increased skin blood flow, as well as behavioral responses. By comparison, preoptic cooling evokes heat-production responses, including shivering and nonshivering thermogenesis, and heat-retention responses, including cutaneous vasoconstriction and behavioral responses.

In amphibians, the neural control of thermoregulation has received limited attention over the years. Although experimental verification is scarce for amphibians, the rostral brain stem has been suggested to be an important site for the regulation of T_b based on thermal stimulation of this area in other ectotherm species such as lizards (Hammel et al., 1967), teleost fish (Crawshaw and Hammel, 1971; Hammel et al., 1969; Nelson and Prosser, 1979) and sharks (Crawshaw and Hammel, 1973). In a shuttle box set up, brain stem warming leads these animals to exit from a warm environment earlier and at a lower T_b than they would normally do. Conversely, brain-stem cooling produces the opposite response. Moreover, heating the rostral brain stem at high ambient temperatures can transiently increase the rate of evaporative water loss in turtles (Morgareidge and Hammel, 1975).

Temperature-sensitive neurons have also been reported to exist in the POA of some ectotherm species such as fish (Nelson and Prosser, 1981a,b) and lizards (Cabanac et al., 1967), and also of birds (Nakashima et al., 1987). From these findings, it has been inferred that centrally located temperature-sensitive neurons are components of a thermoregulatory system in all vertebrates. However, more studies are needed to verify the existence of this sort of neuron in amphibians.

The role of the hypothalamus in the behavioral thermoregulation of ectotherms is also supported by studies in which specific brain nuclei were lesioned in fish (Nelson and Prosser, 1979) and lizards (Berk and Heath, 1975). Nelson and Prosser (1979) reported that sunfish (*Lepomis cyanellus*) and goldfish (*Carassius auratus*) show a well-defined preferred T_b, but lesions placed in the medial preoptic region disrupt this behavior and the fish can then be found evenly distributed along the thermal gradient. In the lizard *Dipsosaurus dorsalis*, Berk and Heath (1975) found that specific hypothalamic areas when lesioned had pronounced effects on both high and low T_b during shuttling.

For the amphibians, to our knowledge only one study has reported a role of the hypothalamus in behavioral thermoregulation. After extensive hypothalamic lesions by electric cautery, juvenile bullfrogs (*Rana catesbeiana*) do not

move at all when introduced within a thermal gradient. Lesioned bullfrogs appear alert and jump when prodded but are quiescent when left undisturbed, even when placed at 35°C (Lillywhite, 1971).

In the present study, we observed no effect of electrolytic lesion in the POA on thermoregulation of *B. paracnemis* under euthermic conditions (Fig. 2). Toads were still actively moving (Fig. 2), indicating that thermoregulation was not disrupted after POA lesions. Conversely, Lillywhite (1971) observed that thermoregulation of euthermic frogs was abolished after hypothalamic lesions. This difference might reside in the fact that Lillywhite (1971) performed extensive hypothalamic lesions, whereas in our experiments the lesions were discrete and restricted to the POA (Fig. 1). In agreement with our observation, as reviewed by Boulant (2000), the compilation of all studies about lesions in the rostral hypothalamus of mammals suggests that no single neural area acts as the center for thermoregulation. Rather, there appears to be a hierarchy of structures extending through the hypothalamus, brain stem and spinal cord. When the nervous system is intact, the role of the higher structures (i.e. the preoptic region) becomes apparent. At least in mammals, the strongest evidence of the importance of the preoptic region in thermoregulation comes from studies involving direct thermal stimulation of this area (Boulant, 2000). This type of investigation has never been carried out in amphibians.

Role of POA in the behavioral fever induced by LPS

It is interesting to note that although lesion in the POA of toads did not result in a loss of thermoregulatory capabilities (Fig. 2), LPS-induced behavioral fever was completely abolished (Fig. 3). Moreover, lesions outside the POA did not change the febrile response of the animals. These results are in agreement with data about the important role of the POA in mammalian fever (Boulant, 1998, 2000). In these animals, which use autonomic and behavioral mechanisms to regulate T_b, fever is functionally expressed as an increase in metabolic heat production and a decrease in heat loss, besides behavior (Cooper, 1995). It is thought that pyrogens and their mediators elevate the thermoregulatory set point by inhibiting the firing rate of preoptic warm-sensitive neurons (Eisenman, 1969; Matsuda et al., 1992; Shibata and Blatteis, 1991; *cf.* Boulant, 1998, 2000). Therefore, this suppresses heat loss and enhances heat production and heat-retention responses, and so fever occurs (*cf.* Boulant, 2000). Our results concerning febrile toads, which thermoregulate primarily by behavior, represent new data favoring the notion that the POA is a site involved in the thermoregulatory set point rise during fever, regardless of the sort of thermoeffector mechanisms present in the tested species.

Behavioral fever in ectotherms was first observed in lizards (Kluger, 1991; Vaughn et al., 1974) and has since been reported in amphibians (Bicego-Nahas et al., 2000; Kluger, 1977; Myhre et al., 1977), fish and even some invertebrates (*cf.* Kluger, 1991), indicating that fever has an ancient phylogenetic history. As to the mechanisms of fever, we now report for the first time an important role of the POA in the development of fever in toads, indicating a considerable degree of phylogenetic conservation of this site in the CNS involved in fever among vertebrates.

Perspectives

Since the classic 1938 paper by Magoun et al. (*cf.* Boulant, 2000) suggesting that the POA is a thermosensitive site in the CNS, a growing body of evidence has confirmed this notion in a wide variety of vertebrate species ranging from fish to mammals and birds. It is currently accepted that, in mammals, a balance between warm-sensitive and temperature-insensitive neurons in the POA determines the thermoregulatory set point. Accordingly, endogenous pyrogens such as interleukin 1 (Shibata and Blatteis, 1991) and prostaglandin E_2 (Matsuda et al., 1992) have been shown to inhibit the sensitivity of warm-sensitive neurons to temperature, a response that is in agreement with the increased thermoregulatory set point during fever (Boulant, 1998).

However, even though fever has been reported to be a response that is extremely widespread among taxa (Kluger, 1991), the role of the POA in the febrile response of ectotherm species has received no attention. We now add data showing that the POA is essential for fever development in an ectotherm species that thermoregulates primarily behaviorally, supporting the notion that the POA is a site involved in the thermoregulatory set-point rise during fever. This evidence favors the view that the mechanisms responsible for fever may have an ancient phylogenetic history. In fact, fever in ectotherms has already been suggested to involve endogenous pyrogens (Myhre et al., 1977) and prostaglandin (Bicego et al., 2002; Hutchison and Erskine, 1981) as pyretic mediators, and the vasopressin analogue arginine vasotocin as an antipyretic molecule (Bicego-Nahas et al., 2000). Nevertheless, data are still lacking regarding the mechanisms involved in the febrile response of ectotherm species to support this assumption. Further experiments are needed to determine how exogenous pyrogens are sensed by ectotherms, how endogenously produced pyrogens signal the brain to produce fever and, once the brain is signalled, what neural pathways are responsible for the effector response.

This work was supported by Fundaçao de Amparo a Pesquisa do Estado de Sao Paulo (FAPESP), Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq). K.C.B. was supported by FAPESP. We thank Rubens Mello and Mauro F. C. Silva for histological preparations.

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