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Caloric stimulation of ampullar receptors: a new method to produce mechanically-evoked responses in frog semicircular canals

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Abstract

A microthermistor positioned close to the exposed posterior semicircular canal in isolated labyrinth preparations of the frog was used to stimulate the sensory organ. Our results indicated that, depending on the position of the heater, the induced endolymphatic convection currents may result in either excitatory or inhibitory cupular deflections and thus in a modulation of ampullar receptor resting activity. Other possible thermal-dependent mechanisms, such as a direct action of the stimulus on vestibular sensors or endolymphatic volume changes, had, in the present experimental conditions, a minor role. Caloric stimulation could therefore represent a novel method to stimulate the semicircular canals 'in situ'.

Keywords: Frog; Vestibular receptors; Semicircular canal; Caloric stimulation; Thermoconvective currents

1. Introduction

According to thermoconvective theory first proposed by Barany (1906), the irrigation of the external ear canal with cool or warm water produces a gravity-dependent transcupular pressure difference, resulting in utriculofugal or utriculopetal deflections of the lateral semicircular canal cupula, which, by modifying ampullar receptor activity (see Kandel et al., 1994 and Guth et al., 1998 for a review), induce the well-known caloric nystagmus. Caloric tests are normally used to validate a diagnosis of asymmetric functioning of vestibular system (Jacobson et al., 1993).

The goal of the present study is to describe and present a method to generate controlled thermally-induced cupular deflections and, therefore, controlled responses of ampullar receptors to caloric stimuli.

This kind of stimulation could allow not only the activation 'in situ' of the semicircular canals but also substitute for other procedures of mechanical stimulation requiring more complex (by producing fluid flows inside the canal; Valli and Zucca, 1977; Valli et al., 1990) or expensive (by using turn-tables; Dickman and Correia, 1989; Rossi et al., 1994; Soto et al., 1994) machinery.

Convective currents, in the case of the caloric testing, are believed to be the main, but not the sole mode of activation of vestibular receptors. In fact, both a direct thermal action on vestibular sensory units and endolymphatic volume changes are claimed to play a role in receptor responses to caloric stimuli (Sherer and Clarke, 1985; Stahle, 1990; Gentine et al., 1990, 1991a,b,c). However, in the experimental conditions adopted in the present study, it can be demonstrated that the importance of these non-thermoconvective mechanisms is negligible and that ampullar receptors are mainly stimulated by thermically-induced transcupular pressure differences.

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The role of non-thermoconvective mechanisms might be an interesting starting point for a future study but is not in line with the aim of the present study.

2. Materials and methods

Experiments were carried out on whole labyrinth preparations isolated from frogs previously anaesthetized by immersion in 0.1% 3-aminobenzoic acid ethyl ester methane sulfonate solution (MS-222, Sandoz).

After decapitation, the lower jaw was removed and the head cut in half. The bony temporal portion containing the right labyrinth was isolated by removing both the surrounding part of the skull and the brain stem. The otic capsule was opened and the posterior canal together with its ampullary nerve micro-dissected free. The preparation was then transferred into a 50 ml perspex chamber filled with artificial perilymph (composition: NaCl 113 mM; KCl 2.5 mM; $NaHCO_3$ 1.2 mM; NaH_2PO_4 0.17 mM; $CaCl_2$ 1.8 mM; glucose 5.5 mM; pH 7.3) and positioned at the center of the chamber so that the posterior canal lay in the vertical plane (Fig. 1). Multiunit nerve firing rate (Nfr), recorded from the posterior ampullar nerve by a suction electrode, was measured using a window discriminator and a frequency-to-voltage converter.

2.1. Thermal stimulation of ampullar receptors

2.1.1. Thermal stimulation apparatus

The apparatus for thermal stimulation of the frog labyrinth consisted of a heating probe (heater) and a current generator. The heater was built using a miniature NTC (negative temperature coefficient) resistor (diameter 1.5 mm, length 5 mm), sealed in a glass tube. The characteristics of the thermistor were:

Rbead (25°C): 1000 ohm $\pm$ 20%
Dissipation constant: 0.75 mW/°C
Thermal time constant: 5 s
B constant: 2910 K $\pm$ 3%.

Rbead indicates the nominal resistance of the resistor at 25°C; the combination between the dissipation constant and time constant indicates the time required to reach thermal steady state conditions. For example, at 25°C, a tension of 0.87 V applied to the thermistor will produce an increase of 1°C in 5 s. B constant indicates the characteristic temperature constant (K) of the resistor.

The heater was driven by a current generator controlled by a PC through its parallel port, and equipped with an in-house software program. Current intensity could be manually set within the range 0–20 mA.

The personal computer allowed control, through an in-house QUICKBASIC program (V. 4.50), of the duration, lag and number of stimulus pulses (Appendix A). The program could drive two current generators, and therefore two heaters, at the same time. This allowed the experimenters to stimulate either different points of the same sensory organ or different sensory organs.

Fig. 2 illustrates the constant current generator. The current we used (0–20 mA) produced a power dissipation in the thermistor in the range 0–120 mW, corresponding to a temperature increase of the liquid-bathed thermistor ranging from 0 to 65°C.

Fig. 3 shows the liquid-bathed thermistor heating versus current intensity with 40 s long heating pulses

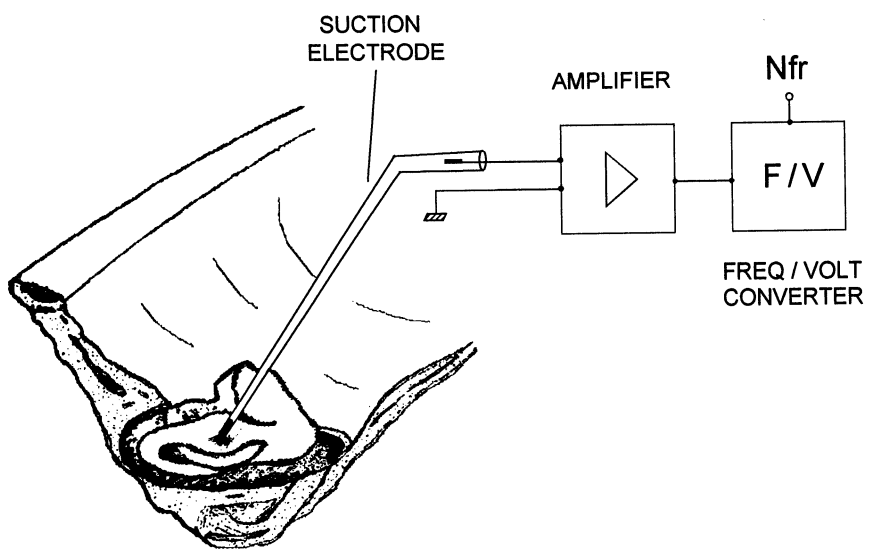

Fig. 1. Schematic representation of the experimental set-up for recording multiunit nerve firing rate (Nfr) from the ampullar nerve of the frog posterior canal.

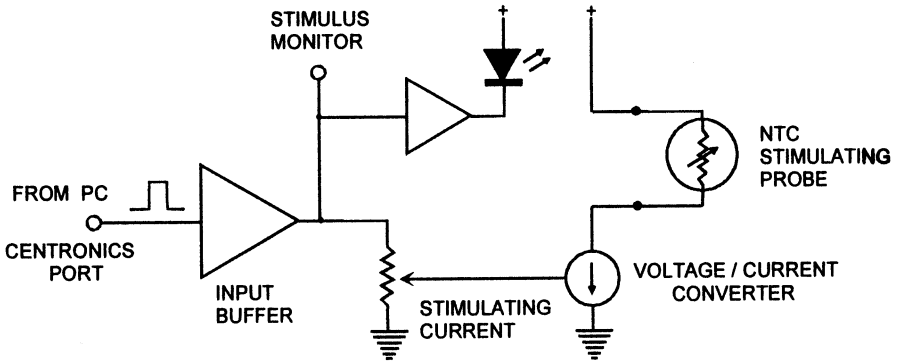

Fig. 2. Scheme of the electronic apparatus used to vary the current intensity across the microthermistor, and therefore the thermal stimulus intensity delivered to the ampullar receptors.

(more than sufficient to reach thermal equilibrium; Fig. 5). To evaluate the temperature increase of the liquid bathing the heater (and therefore of the semicircular canal wall) as a function of stimulus current we used a microthermometer, made by a second NTC thermistor, the mass of which and thermal time constant were much smaller than those of the stimulating thermistor.

For currents ranging from 0 to 20 mA and for a stimulus duration of 40 s, the temperature increases in the liquid contacting the probe are displayed in Fig. 4. Fig. 5 shows the time course of the liquid temperature in response to a 14 mA current applied for 40 s. It may be noted that a constant temperature is reached after about 5 s of current application.

We also measured the temperature changes in the bath at various distances from the heater, keeping current intensity and duration constant (14 mA, 40 s). It may be seen (Fig. 6) that, by increasing the distance between the heater and the microthermometer, the temperature decreased in an exponential way. At about 1.6–1.8 mm from the heater no change in the liquid temperature could be detected.

A digital thermometer placed in the bath at a certain distance from the heather (more than 2 mm) indicated that, in the present experimental conditions, no change in the bath temperature could be detected even after several hours of stimulation.

Regarding the arrangement of the preparation, the right half head of the frog was fixed at the center of the perspex chamber in such a way that the posterior canal lay in the vertical plane (Fig. 7). The heater was positioned, as a rule, at about 10 μm from the canal wall, either in (B), i.e. close to the crus commune or in (A). Thus they were arranged symmetrically with regard to the ampulla of the posterior canal (see scheme in Fig. 7).

The present experiments were performed in accordance with the guidelines of the Declaration of Helsinki.

3. Results

As shown in Fig. 8, the firing rate of ampullar receptors was extremely sensitive to temperature changes in the fluid contacting the canal walls. Temperature changes as low as 0.08°C were sufficient to produce distinct changes in receptor resting discharge and responses saturated for temperature changes higher than 1°C. It may be noted that when the heater was positioned in (B), i.e. close to the crus commune, a condition in which utriculofugal (excitatory) endolymphatic flows would be generated, a prompt increase in nerve firing rate (Nfr) was observed. Especially for the highest stimuli tested (1–1.50°C), this Nfr enhancement soon decreased and, in about 30–40 s, gave rise to an undershoot lasting 40–50 s. At the removal of the excitatory stimulus the sensory discharge was inhibited and recovered its resting value in about 2 min.

When the heater was positioned in (A), i.e. in a position in which utriculopetal (inhibitory) flows were generated, the firing rate was clearly decreased and, for the highest stimuli tested, nearly zeroed for about 15–20 s. Then Nfr slowly increased but without recovering its resting level within the stimulation period (2 min). At the removal of the inhibitory stimulus, a rebound discharge was observed, the intensity and duration of which were dependent on stimulus strength.

Fig. 9 refers to an experiment in which a saturating excitatory stimulus (10 mA, 1.10°C) was applied every 15 min to the same preparation over a period of 8 h. It may be seen that only slight changes in both resting and thermally-evoked activity could be detected as a function of survival time. This indicates that this kind of stimulation is well-tolerated by the sensory organ.

The results described in Fig. 8 clearly demonstrate that, according to the position of the heater, it is possible to obtain both excitatory or inhibitory responses of ampullar receptors. It follows that by employing two heaters, one located in (A) and the other in

(B), and a suitable program of stimulation where the stimulus of one heater is delayed with respect to the other, it is possible to obtain a succession of excitatory and inhibitory cupular deflections and therefore 'sinusoidal-like' responses of ampullar receptors to caloric stimuli (Fig. 10).

4. Discussion

The findings of the present study clearly demonstrate that ampullar receptors can be thermally stimulated (activated or inhibited) by means of microheaters placed in the vicinity of the sensory organ. The results referred to experiments carried out on posterior semicircular canals but, with suitable microdissection and positioning of the heater, anterior and lateral canals can be thermally stimulated as well (data not shown).

The experiments have also shown that this kind of stimulation is perfectly tolerated by the preparation. In fact ampullar receptors could respond to thermal stimuli with constant and repeatable responses for 8 h.

Caloric stimulation of semicircular canals therefore can be usefully employed to induce mechanically-evoked responses of ampullar receptors and, if particular patterns of stimulation are not required, can replace other methods of stimulation which may be more complicated, damaging or expensive. This method might be particularly suitable in experiments in which intracellular recordings from single fibres are required. In fact, during caloric stimulation of ampullar receptors, no movements of the preparation are produced and this greatly facilitates the maintaining of the impalement for a longer time.

Regarding the mechanism of hair cell activation, our results suggest that thermoconvective endolymphatic currents (producing steady transcupular pressure differences and, therefore, steady cupular deflections) are involved. Other possible mechanisms such as a direct effect of the temperature on vestibular sensors (Zucca et al., 1983a,b; Rossi et al., 1995; Zenner and Zimmermann, 1995) or endolymphatic volume changes (Gentine et al., 1991b) play, if any, a minor role. In fact the heater was placed about 2 mm from the sensory epithelium (Fig. 7). At this distance, even the highest stimulus tested was unable to produce measurable thermal changes at the level of the ampulla (Fig. 6).

Regarding the importance of the expansion of the endolymph, it may be calculated that, within the range tested ($\Delta T = 0$–1.5°C), the highest hydrostatic pressure generated by this mechanism (Gentine et al., 1991b) is about 10^{-1} Pa. In a previous study we demonstrated that hydrostatic pressure differences of at least 0.25 mmH_2O between the endolymph and the perilymph (about 2.5 Pa) are needed to affect the activity of vestibular sensors (Zucca et al., 1991). This pressure therefore is about 25 times lower than that needed to modify ampullar receptor activity.

The hypothesis that the main effect of thermal stimuli was to produce transcupular pressure differences is further supported by the fact that the time course of thermally-evoked responses was very similar to that observed in isolated semicircular canals after application of step mechanical stimuli of the same duration (Zucca et al., 1993).

Appendix A. Listing of the program

'Main Program "ESE5A.BAS"

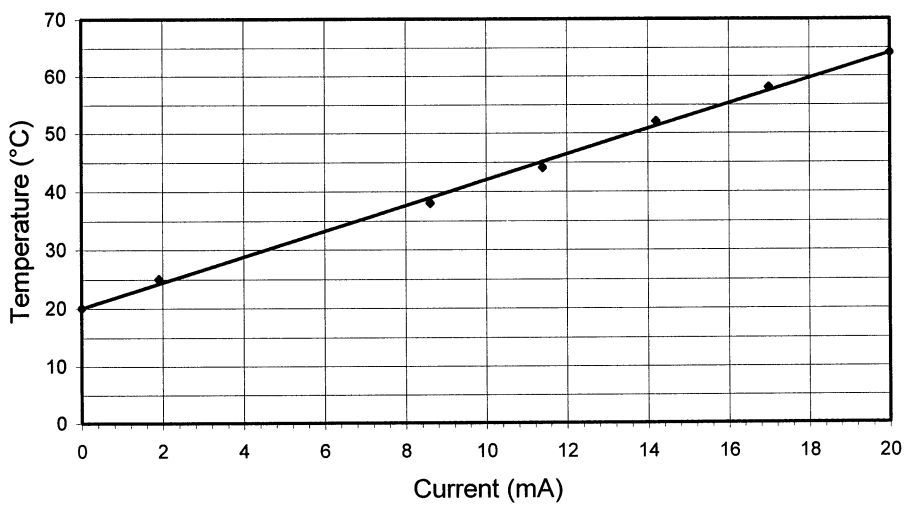

Fig. 3. Relation between the current across the thermistor and the temperature of the thermistor itself. The experimental points used to calculate the regression curve were obtained using the thermistor temperature–resistance relation, given by the builder of the microthermistor itself. The relation is described by the straight line of equation: $y = 2.2013x + 20.012$ ($R^2 = 0.9978$).

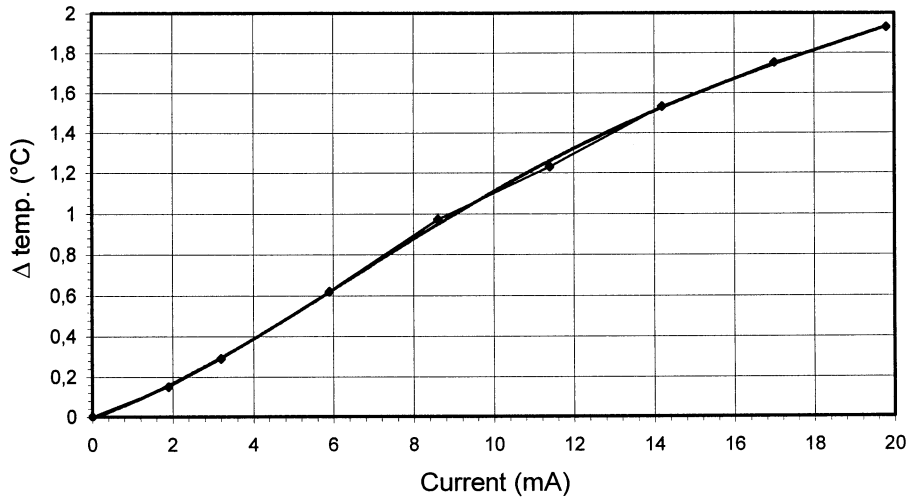

Fig. 4. Relation between the current across the thermistor and temperature changes (ΔT) in the fluid bathing the heater. The experimental points used to obtain the regression curve were measured by means of a microthermometer positioned about 10 μm from the heater. The relation is described by the equation: $y = 0.003x^4 - 0.0082x^3 + 0.0557x^2 + 0.1826x - 0.2325$ ($R^2 = 0.9997$).

```
DECLARE SUB contemp ()
DECLARE SUB dopinv ()
DECLARE SUB Inibitorio ()
DECLARE SUB eccitatorio ()
DECLARE SUB doppio ()
SCREEN 0
OUT 888, 0

menu:
CLS
LOCATE 2, 5: PRINT CHR$(201);: PRINT
STRING$(70, 205);: PRINT CHR$(187);
FOR x = 3 TO 20
LOCATE x, 5: PRINT CHR$(186);: LOCATE x, 76:
PRINT CHR$(186);
NEXT x
LOCATE 21, 5: PRINT CHR$(200);: PRINT
STRING$(70, 205);: PRINT CHR$(188);
LOCATE 5, 25: COLOR 0, 7: PRINT
"LABYRINTH THERMAL STIMULATION";:
COLOR 7, 0
LOCATE 7, 15: COLOR 15: PRINT "1";: COLOR
7: PRINT "–Excitatory stimulus ↑↑↑"
LOCATE 9, 15: COLOR 15: PRINT "2";: COLOR
7: PRINT "–Inhibitory stimulus ↓↓↓"
LOCATE 11, 15: COLOR 15: PRINT "3";: COLOR
7: PRINT "–Excitatory + inhibitory stimuli ↑↓↑↓"
LOCATE 13, 15: COLOR 15: PRINT "4";: COLOR
7: PRINT "–Inhibitory + excitatory stimuli ↓↑↓↑"
LOCATE 15, 15: COLOR 15: PRINT "5";: COLOR
7: PRINT "–Simultaneous stimuli ϕϕϕϕ"
LOCATE 17, 15: COLOR 15: PRINT "6";: COLOR
7: PRINT "-Exit"
LOCATE 19, 40: INPUT "Stimulus?"; sc%
SELECT CASE sc%
CASE 1: CALL eccitatorio
CASE 2: CALL Inibitorio
CASE 3: CALL doppio
CASE 4: CALL dopinv
CASE 5: CALL contemp
CASE 6: CLS: END
CASE ELSE: PRINT: PRINT "print a number (1–
6)"
END SELECT
GOTO menu
END

SUB contemp
DEFINT A-M
parcon:
CLS
LOCATE 2, 24: COLOR 0, 7: PRINT "SIMULTA-
NEOUS STIMULI": COLOR 7, 0
LOCATE 4, 10
INPUT "Stimulus duration (sec)"; ds1
```

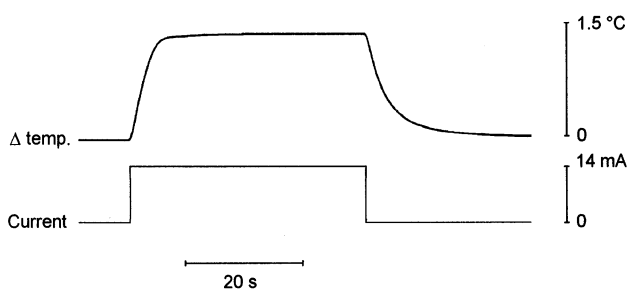

Fig. 5. Comparison between the time course of the current across the heater and the temperature changes (ΔT) in the surrounding fluid.

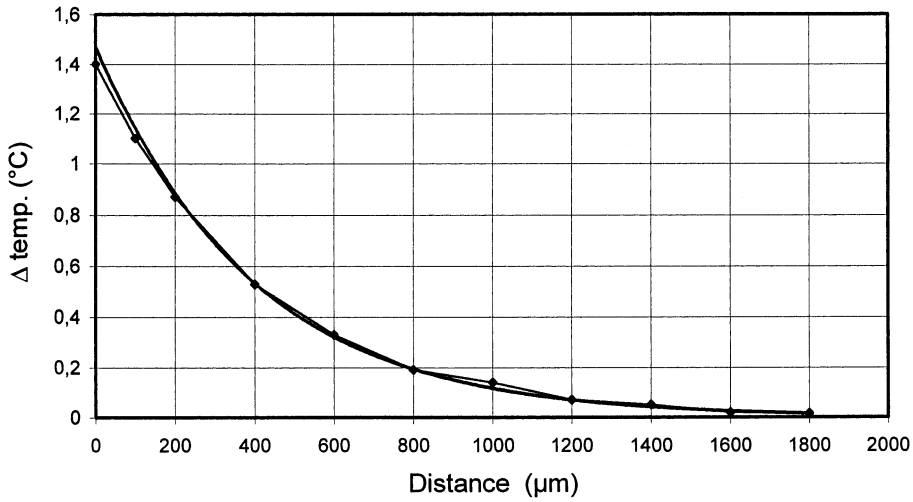

Fig. 6. Temperature changes (ΔT) at increasing distances from the heater. Stimulus parameters: 14 mA; 40 s. The curve fitting the experimental points was described by the exponential equation: $y = 1.47e^{-0.0025x}$ ($R^2 = 0.995$).

```
LOCATE 6, 10
INPUT "Cycles' number"; nc
LOCATE 8, 10
INPUT "Delay between two stimuli (sec)"; rs1
LOCATE 10, 30: COLOR 27, 0: PRINT "for stimulation...";: COLOR 7, 0
LOCATE 25, 5: PRINT " < ESC > to change data- main menu";
tasti:
kbd$ = INKEY$
  IF kbd$ = ""THEN GOTO tasti
  IF kbd$ = CHR$(0) + CHR$(68) THEN GOTO incont
  IF kbd$ = CHR$(27) THEN GOTO parcon
  IF kbd$ = CHR$(0) + CHR$(59) THEN GOTO fin
  GOTO tasti
END
incont:
LOCATE 10, 30: COLOR 7, 0: PRINT"
LOCATE 12, 15: PRINT "—Start stimulation—"
FOR b = 1 TO nc
PRINT "Stimulus"; b
tim1 = TIMER
tim2 = tim1 + ds1
OUT 888, 192
DO UNTIL TIMER > = tim2
LOOP
OUT 888, 0
rit1 = TIMER
rit2 = rit1 + rs1
DO UNTIL TIMER > = rit2
LOOP
NEXT b
PRINT "—End stimulation—": PRINT
PRINT "for stimulation..."
LOCATE 25, 3: PRINT "main menu";
opzio:
kbd$ = INKEY$
  IF kbd$ = ""THEN GOTO opzio
  IF kbd$ = CHR$(0) + CHR$(68) THEN GOTO parcon
  IF kbd$ = CHR$(0) + CHR$(59) THEN GOTO fin
END
fin:
CLS
END SUB


DEFSNG A-M
SUB dopinv
DEFINT A-M
pardi:
CLS
LOCATE 2, 20: COLOR 0, 7: PRINT "INHIBITORY + EXCITATORY STIMULI": COLOR 7, 0
LOCATE 5, 10
INPUT "Inhibitory stimulus duration (sec)"; di1
LOCATE 6, 10
INPUT "Excitatory stimulus duration (sec)"; de1
LOCATE 7, 10
INPUT "Cycles' number"; nc
LOCATE 8, 10
INPUT "Delay after the inhibitory stimulus (sec)"; ri1
LOCATE 9, 10
INPUT "Delay after the excitatory stimulus (sec)"; re1
LOCATE 10, 30: COLOR 27, 0: PRINT "for stimulation...";: COLOR 7, 0
```

```
LOCATE 25, 5: PRINT " < ESC > to change data-
main menu";
tastt:
kbd$ = INKEY$
  IF kbd$ = ""THEN GOTO tastt
  IF kbd$ = CHR$(0) + CHR$(68) THEN GOTO
  indi
  IF kbd$ = CHR$(27) THEN GOTO pardi
  IF kbd$ = CHR$(0) + CHR$(59) THEN GOTO
  finn
  GOTO tastt
END
indi:
LOCATE 10, 30: COLOR 7, 0: PRINT"";
LOCATE 12, 15: PRINT "—Start stimulation—"
FOR b = 1 TO nc
PRINT "Stimulus"; b
tim3 = TIMER
tim4 = tim3 + di1
OUT 888, 64
DO UNTIL TIMER > = tim4
LOOP
OUT 888, 0
rit3 = TIMER
rit4 = rit3 + ri1
DO UNTIL TIMER > = rit4
LOOP
tim1 = TIMER
tim2 = tim1 + de1
OUT 888, 128
DO UNTIL TIMER > = tim2
LOOP
OUT 888, 0
rit1 = TIMER
rit2 = rit1 + re1
DO UNTIL TIMER > = rit2
LOOP
NEXT b
PRINT "—End stimulation—": PRINT
PRINT "for stimulation"
LOCATE 25, 5: PRINT "main menu";
opzione:
kbd$ = INKEY$
  IF kbd$ = ""THEN GOTO opzione
  IF kbd$ = CHR$(0) + CHR$(68) THEN GOTO
  pardi
  IF kbd$ = CHR$(0) + CHR$(59) THEN GOTO
  finn
END
finn:
CLS
END SUB

DEFSNG A-M
SUB doppio
DEFINT A-M
pardop:
CLS
LOCATE 2, 20: COLOR 0, 7: PRINT "EXCITA-
TORY + INHIBITORY STIMULI": COLOR 7, 0
LOCATE 5, 10
INPUT "Excitatory stimulus duration (sec)"; de1
LOCATE 6, 10
INPUT "Inhibitory stimulus duration (sec)"; di1
LOCATE 7, 10
INPUT "Cycles' number"; nc
LOCATE 8, 10
INPUT "Delay after the excitatory stimulus (sec)";
re1
LOCATE 9, 10
INPUT "Delay after the inhibitory stimulus (sec)";
ri1
```

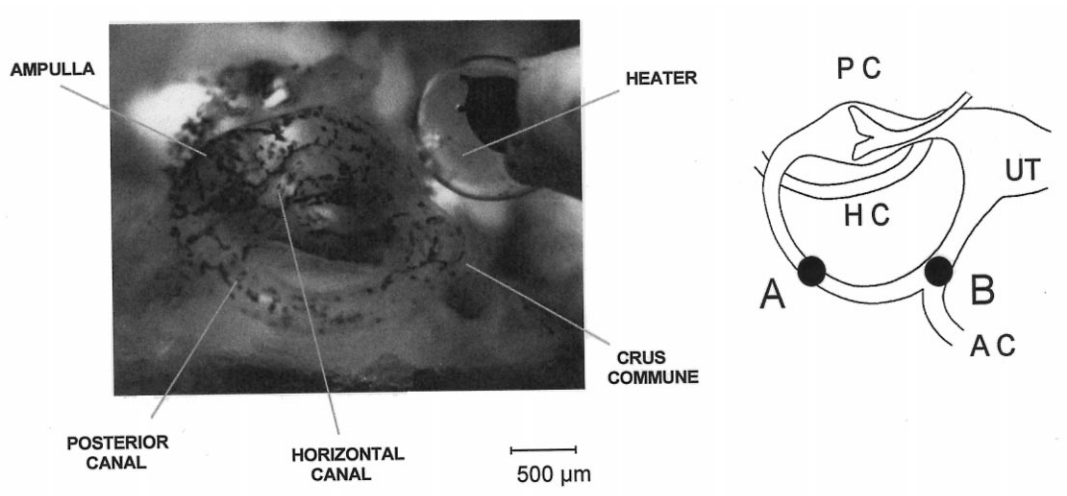

Fig. 7. Microphotography showing both the preparation and the heater which, as indicated by the inset, was usually placed in (A) or in (B) (see text).

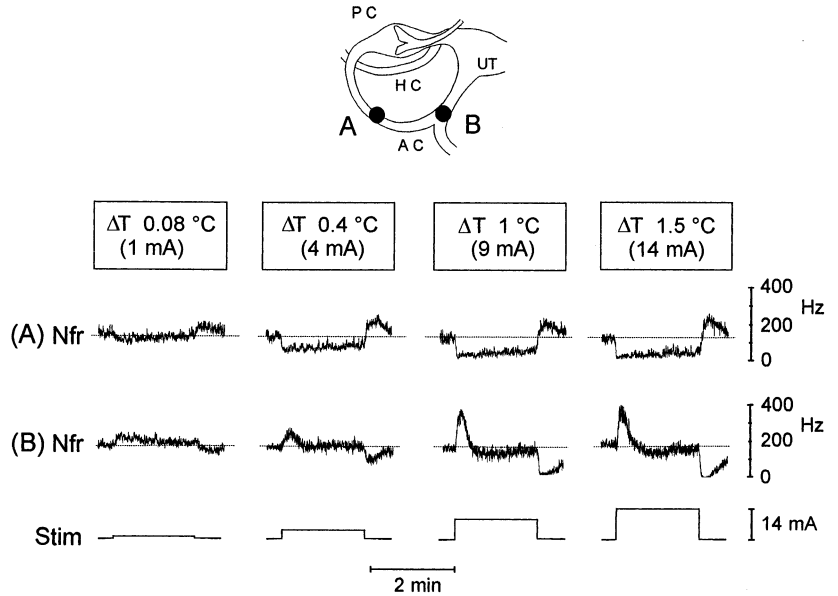

Fig. 8. Effects of thermal stimuli (14 mA; 2 min) applied in (A) or in (B) on the firing rate (Nfr) recorded from the ampullary nerve of the posterior canal. Dotted lines indicate the level of the resting activity.

```
LOCATE 10, 30: COLOR 27, 0: PRINT "for stimulation...";: COLOR 7, 0
LOCATE 25, 5: PRINT " < ESC > to change data-main menu";
tast:
kbd$ = INKEY$
  IF kbd$ = ""THEN GOTO tast
  IF kbd$ = CHR$(0) + CHR$(68) THEN GOTO indop
  IF kbd$ = CHR$(27) THEN GOTO pardop
  IF kbd$ = CHR$(0) + CHR$(59) THEN GOTO finee
  GOTO tast
END
indop:
LOCATE 10, 30: COLOR 7, 0: PRINT "";
LOCATE 12, 15: PRINT "—Start stimulation—"
FOR b = 1 TO nc
PRINT "Stimulus"; b
tim1 = TIMER
tim2 = tim1 + de1
OUT 888, 128
DO UNTIL TIMER > = tim2
LOOP
OUT 888, 0
rit1 = TIMER
rit2 = rit1 + re1
DO UNTIL TIMER > = rit2
LOOP
tim3 = TIMER
tim4 = tim3 + di1
OUT 888, 64
DO UNTIL TIMER > = tim4
LOOP
OUT 888, 0
rit3 = TIMER
rit4 = rit3 + ri1
DO UNTIL TIMER > = rit4
LOOP
NEXT b
PRINT "—End stimulation—": PRINT
PRINT "for stimulation..."
LOCATE 25, 5: PRINT "main menu";
opzi:
kbd$ = INKEY$
  IF kbd$ = ""THEN GOTO opzi
  IF kbd$ = CHR$(0) + CHR$(68) THEN GOTO pardop
  IF kbd$ = CHR$(0) + CHR$(59) THEN GOTO finee
END
finee:
CLS
END SUB


DEFSNG A-M
SUB eccitatorio
DEFINT A-M
parecc:
CLS
LOCATE 2, 24: COLOR 0, 7: PRINT "EXCITATORY STIMULUS": COLOR 7, 0
LOCATE 4, 10
```

```
INPUT "Excitatory stimulus duration (sec)"; d1
LOCATE 6, 10
INPUT "Number of stimuli"; n1
LOCATE 8, 10
INPUT "Delay between stimuli (sec)"; r1
LOCATE 10, 30: COLOR 27, 0: PRINT "for stimulation...";: COLOR 7, 0
LOCATE 25, 5: PRINT " < ESC > to change data-main menu";
tasto:
kbd$ = INKEY$
  IF kbd$ = ""THEN GOTO tasto
  IF kbd$ = CHR$(0) + CHR$(68) THEN GOTO inecc
  IF kbd$ = CHR$(27) THEN GOTO parecc
  IF kbd$ = CHR$(0) + CHR$(59) THEN GOTO fine
  GOTO tasto
END
inecc:
LOCATE 10, 30: COLOR 7, 0: PRINT "";
LOCATE 12, 15: PRINT "—Start stimulation—"
  FOR a = 1 TO n1
  PRINT "Stimulus"; a
  tim1 = TIMER
  tim2 = tim1 + d1
  OUT 888, 128
    DO UNTIL TIMER > = tim2
    LOOP
  OUT 888, 0
  rit1 = TIMER
  rit2 = rit1 + r1
    DO UNTIL TIMER > = rit2
    LOOP
  NEXT a
PRINT "—End stimulation—": PRINT
PRINT "for stimulation"
LOCATE 25, 3: PRINT "main menu";
scelta:
kbd$ = INKEY$
  IF kbd$ = ""THEN GOTO scelta
  IF kbd$ = CHR$(0) + CHR$(68) THEN GOTO parecc
  IF kbd$ = CHR$(0) + CHR$(59) THEN GOTO fine
END
fine:
CLS
END SUB


DEFSNG A-M
SUB Inibitorio
DEFINT A-M
parin:
CLS
LOCATE 2, 24: COLOR 0, 7: PRINT "INHIBITORY STIMULUS": COLOR 7, 0
LOCATE 4, 10
INPUT "Inhibitory stimulus duration (sec)"; d3
LOCATE 6, 10
INPUT "Number of stimuli"; n3
LOCATE 8, 10
INPUT "Delay between stimuli (sec)"; r3
LOCATE 10, 30: COLOR 27, 0: PRINT "for stimulation...";: COLOR 7, 0
LOCATE 25, 5: PRINT " < ESC > to change data-main menu";
tas:
kbd$ = INKEY$
  IF kbd$ = ""THEN GOTO tas
  IF kbd$ = CHR$(0) + CHR$(68) THEN GOTO inin
```

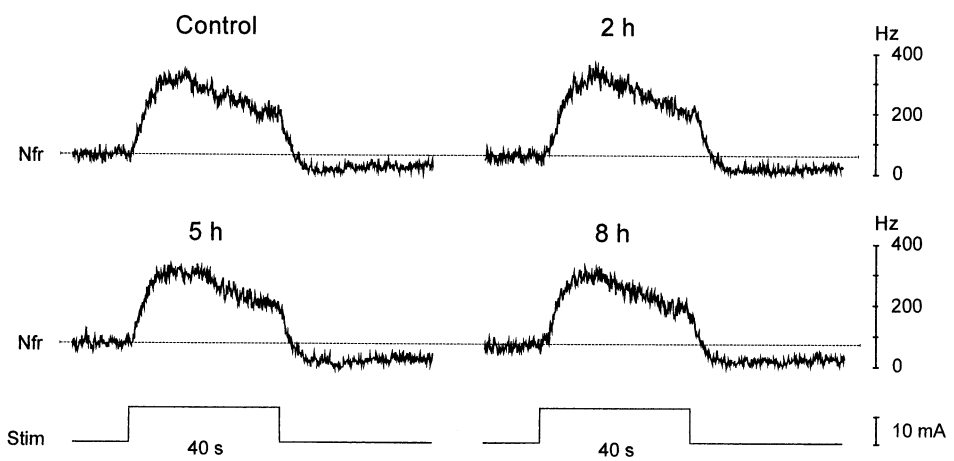

Fig. 9. Nerve firing rate (Nfr) recorded from the ampullar nerve as a function of time. The preparation was thermally stimulated (10 mA; 40 s) every 15 min over a period of 8 h. Dotted lines indicate the level of the resting activity.

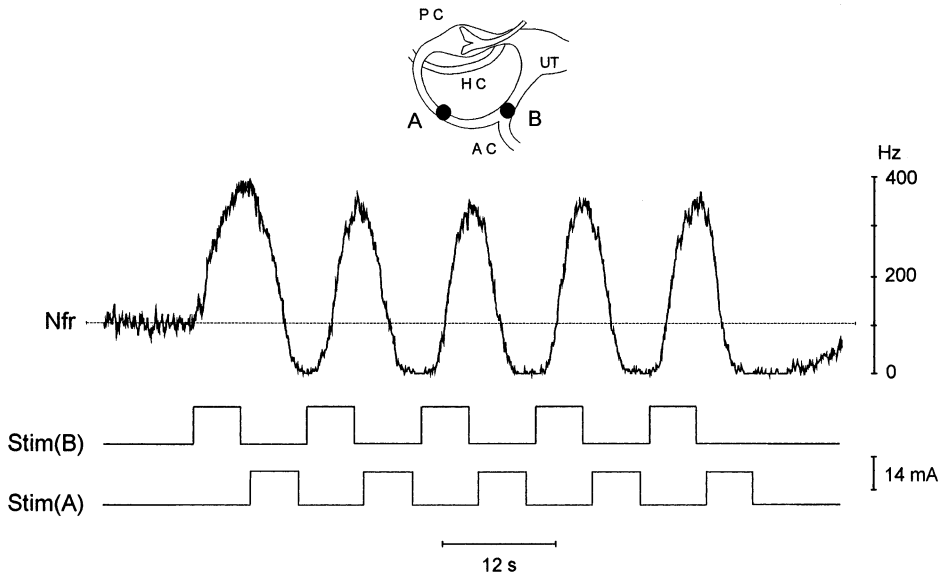

Fig. 10. Sinusoidal-like responses of ampullar receptors to suitable caloric stimuli applied alternatively in (B) and in (A). Dotted lines indicate the level of the resting activity.

```
    IF kbd$ = CHR$(27) THEN GOTO parin
    IF kbd$ = CHR$(0) + CHR$(59) THEN GOTO
    finne
    GOTO tas
END
inin:
LOCATE 10, 30: COLOR 7, 0: PRINT "";
LOCATE 12, 15: PRINT "—Start stimulation—"
    FOR a = 1 TO n3
    PRINT "Stimulus"; a
    tim3 = TIMER
    tim4 = tim3 + d3
    OUT 888, 64
        DO UNTIL TIMER > = tim4
        LOOP
    OUT 888, 0
    rit3 = TIMER
    rit4 = rit3 + r3
        DO UNTIL TIMER > = rit4
        LOOP
    NEXT a
PRINT "—End stimulation—": PRINT
PRINT "for stimulation..."
LOCATE 25, 5: PRINT "main menu";
scel:
kbd$ = INKEY$
    IF kbd$ = ""THEN GOTO scel
    IF kbd$ = CHR$(0) + CHR$(68) THEN GOTO
    parin
    IF kbd$ = CHR$(0) + CHR$(59) THEN GOTO
    finne
END
finne:
CLS
END SUB
```

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