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Short communication

Conjunctivally administered NGF antibody reduces pain sensitivity and anxiety-like behavioral responses in aged female mice

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ABSTRACT

This study reports that peripheral administration of Nerve Growth Factor antibodies (ANA) affects behavior in aged female CD-1 mice. ANA increased the propensity of mice to stay and perform behaviors in the anxiogenic open arms of the maze, lowered pain sensitivity and reduced behavioral flexibility in a Morris water maze task, also reducing ChAT immunoreactivity in the basal forebrain. These findings support the hypothesis that topical eye application can represent an alternative route for delivering biologically active compounds into the brain allowing studying the role of NGF on brain cell function.

Nerve Growth Factor (NGF) is a neurotrophin playing a critical trophic role on forebrain cholinergic neurons (FCN) that degenerate during brain aging and neurodegenerative disorders [1,2]. We have recently shown that NGF administered as a collyrium can affect retina, optic nerve cells, as well as neurons, suggesting that ocular application of high molecular weight proteins can allow their delivery to the brain [3]. It is possible to hypothesize that by inhibiting the biological activity of the endogenously produced NGF, the physiological function of this trophic factor might become more evident. In particular, decreased cholinergic trophism related to NGF availability should be reflected in changes in anxiety and cognitive behavior which are under cholinergic control [4,5]. The study was performed in aged subjects in order to unveil possible differences in NGF function at a time (aging) when cholinergic trophism is impaired and lack of NGF might have a greater impact on brain plasticity. To this purpose, in this study we tested for the first time the effects of ocular application of an anti-NGF antibody (ANA) on NGF-producing and NGF-responsive neurons. Results show that ANA ocular administration affected animals' anxiety and pain sensitivity as well as behavioral flexibility in a spatial memory test in addition to reducing choline acethyl-transferase (ChAT) staining in forebrain neurons.

Experimental subjects were 13 female CD-1 mice (24-month-old) purchased from a commercial breeder (Charles River) kept under standard conditions (temperature 21 ± 1 °C, relative humidity 60 ± 10%, lights on from 20:30 to 08:30).

Lyophilized purified ANA, raised in goat, was dissolved in sterile saline at a concentration of 100 μg/ml and 10 μl of this solution were administered as eye drops by means of a micropipette to both eyes 3 time/day for 21 days (chronic treatment, $n = 7$). The control group ($n = 6$) was treated with saline solution with an identical treatment schedule. Body weight was measured on Days 1, 7 and 21.

The day following the end of treatment, mice were tested for anxiety in an elevated-plus maze (EPM) and for pain sensitivity in a hot plate (HP) test. The EPM consisted in a 5 min session and the time spent in the open vs. the closed arms as well as frequency and duration of behaviors performed during the free exploration of the apparatus (grooming and head-dipping) were assessed. As for the HP test (50 ± 0.1 °C, Model D837 Socrel, Comerio, Italy), latency, frequency and duration of forepaw, hindpaw licking and foot-shacking were scored during a 60 s single session. On the following week, cognitive abilities were assessed in the Morris water maze (MWM) spatial navigation task. The maze (a black Plexiglas circular pool 88 cm in diameter and 33 cm in height) was ideally divided into

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four quadrants: a "target" quadrant (where a hidden platform was located during an acquisition phase), a quadrant opposite to this, referred as the "reversal quadrant" (where the platform was moved during a reversal phase), and two remaining quadrants to the left and right side of the target one. A "cued phase" (4 trials with a visible platform) was performed before the learning procedure to test for sensorimotor and motivational factors independent from spatial learning, in addition to assessing for possible significant visual deficits due to ANA administration [6,7]. An acquisition phase (4 days, 3 trials/day), a probe trial (on Day 5) and a reversal phase (3 trials performed on Day 5 starting 1 h after the probe trial) followed; each trial had a cut-off time of 60 s. Latency to reach the platform was measured during acquisition and reversal phases, as well as velocity, cumulative distance and thigmotaxis (for a detailed description of EPM, HP and MWM apparatuses and experimental procedures see [5,8]).

Soon after the last trial of the MWM, 4 subjects for each group of treatment were sacrificed, both hippocampi dissected out and the concentration of NGF in the homogenized tissue was measured with ELISA assays following the instructions provided by the manufacturer (DuoSet ELISA Development kit, "NGF Emax™ Immunoassay System number G7631"). All measurements were run at least in duplicate for each sample [9]. As for the remaining subjects, they were anesthetized, transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4, and brains were removed and used for ChAT immunohistochemistry (see [10]).

All experimental procedures have been carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the Italian legislation (DecretoL.vo 116/92). All efforts were taken to prevent or reduce animal suffering and for limiting the number of experimental subjects.

When collected data followed a normal distribution they were analyzed using analysis of variance (ANOVA) followed by Tukey's test. Repeated measures were taken into account when performing ANOVA for the MWM test. Since latencies in the HP test failed to fit the criterion of a normal distribution, they were analyzed using a non-parametric Mann and Whitney *U*-test.

Treatment with ANA did not affect body weight compared to vehicle [$F(1,33) = 0.325$; $p = 0.5798$]. All subjects appeared healthy notwithstanding the old age.

Results from the EPM indicated a greater propensity for ANA-treated subjects to stay and perform behaviors in the anxiogenic open arms of the maze. More in detail, ANA-treated subjects did not prefer the closed arms as controls did [Percent time, interaction between treatment and zone: $F(1,11) = 5.263$; $p = 0.0425$, Fig. 1A]. When in the open arms, ANA-treated mice performed head-dipping behavior for longer time than controls [duration, $F(1,11) = 5.264$; $p = 0.0424$, Fig. 1B] although not more frequently [frequency, $F(1,11) = 3.472$; $p = 0.0893$]. In addition, they performed grooming behavior for shorter time in the closed arms [duration, $F(1,11) = 5.293$; $p = 0.0420$, Fig. 1C], while frequency did not differ [$F(1,11) = 3.445$; $p = 0.0904$].

In the HP test, ANA-treated subjects were characterized by a decreased pain sensitivity as shown by the lower frequency of foot-shaking [main effect of treatment $F(1,11) = 5.225$; $p = 0.0431$, see Fig. 2], one of the most reliable measure of thermal pain sensitivity in senescent mice [5,11]. However treated mice did not differ from controls in the latency to perform this behavior [$U(6,7) = 8.50$; $p = 0.2332$]. Likewise, no difference was found as a function of treatment for the remaining behavioral items considered in this test [forepaw licking: latency, $U(6,7) = 14.50$; $p = 0.3511$ and $F(1,11) = 0.240$, 0.096; $p = 0.6339$ and $p = 0.7620$, respectively for duration and frequency; hindpaw licking: latency $U(6,7) = 8.50$; $p = 0.0737$ and $F(1,11) = 1.120$, 2.264; $p = 0.3127$, $p = 0.1605$, respectively for duration and frequency].

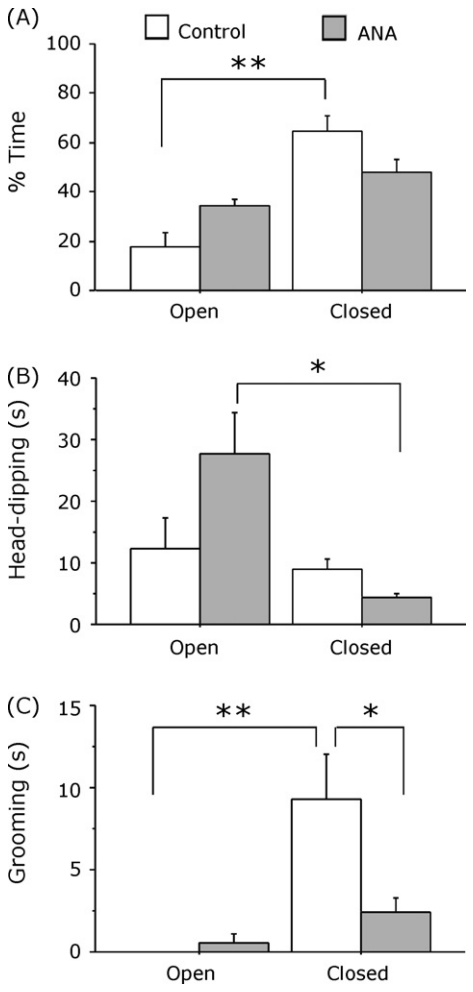

Fig. 1. Behaviors in the open and closed arms of the EPM: (A) ANA-treated subjects showed a lower anxiety as they did not prefer the open arms as did the controls, which spent more time in this part the maze. (B) ANA-treated subjects spent more time performing head-dipping, a form of exploration, in the open arms of the maze. (C) ANA-treated mice performed less grooming behavior than controls in the closed arms. Data shown represent mean + S.E.M.; $n = 6$ for control and 7 for ANA-treated subjects; post hoc comparisons: $^{*}p < 0.05$; $^{**}p < 0.01$.

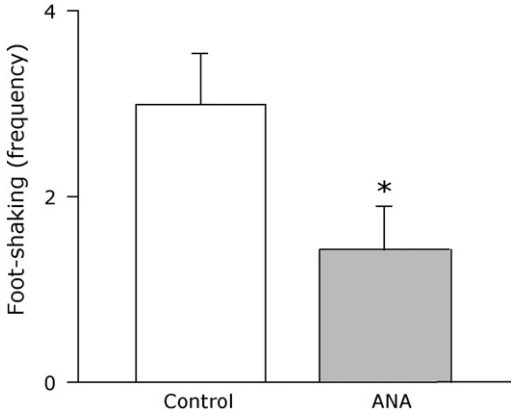

Fig. 2. Behaviors in the HP test: anti-NGF antibody (ANA)-treated subjects showed a lower foot-shaking frequency overall indicating a reduced thermal sensitivity. Data shown represent mean + S.E.M.; $n = 6$ for controls and 7 for ANA-treated subjects; $^{*}p < 0.05$.