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# Nitric oxide is necessary for visual learning in *Octopus vulgaris*

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## SUMMARY

We recently reported that inhibition of nitric oxide synthase (NOS) in *Octopus vulgaris* by intramuscular injections of an analog of L-arginine, N- $\omega$ -nitro-L-arginine methyl ester (L-NAME), blocked touch learning in *Octopus vulgaris*. The inactive enantiomorph (D-NAME), which had no effect on learning, was used for control. We now report that essentially the same procedures block visual learning in this animal. We used a visual paradigm in which the octopus was trained to respond positively to a smooth black plastic ball 2.5 cm diameter and negatively to a similar white ball, or *vice versa*. One set of eight animals was trained to the black ball positive, and another set of six to the white ball positive. Each set was trained at different times by two different trainers. We found that a 1 h pretreatment with the nitric oxide synthase inhibitor L-NAME blocks visual learning in *Octopus vulgaris* in both sets of animals.

## 1. INTRODUCTION

Octopuses have monocular vision and can learn to distinguish between various shapes visually (Muntz *et al.* 1962), for example, between rectangular objects of the same size presented horizontally or vertically and of round objects of different reflectance (e.g. black or white) (Young 1971; Wells 1978). Interestingly, they cannot differentiate between rectangular objects of the same reflectance presented at 45 and 135 degrees to the horizontal (Sutherland 1957; Sutherland 1969). This may relate to the fact that the visual receptors in the retina are oriented in two sets at right angles and that the dendritic fields of the secondary neurons to which the retinal receptor neurons project in the optic lobes are also oriented predominantly at right angles to one another (Young 1971). *Octopus vulgaris* is colour blind (Messenger 1977), although it is reported to be able to distinguish between black, red and white objects on the basis of reflectance differences and background contrast (Fiorito & Scotto 1992).

Blind octopuses cannot differentiate by touch between objects of different shapes presented in different orientations. For instance, whereas the normal animal can differentiate between rectangular shapes of the same size and reflectance presented horizontally or vertically, blinded octopuses cannot make this distinction by touch. This presumably is related to a general lack of proprioceptive sense in octopus (Wells 1978). However, we chose to use smooth black and white balls in the studies reported here. This avoids

any interference from either touch or proprioceptive learning.

Nitric oxide, associated nitrogen oxides and nitrosothiols are produced by the conversion of arginine to citrulline by nitric oxide synthase (NOS), has many functions (Moncada *et al.* 1992; Stamler *et al.* 1992; Schuman & Madison 1994). NOS can be inhibited by several analogs of arginine, including the levorotatory form of the methyl ester of nitroarginine, L-NAME. The other optically active form, D-NAME, is inactive as a NOS inhibitor. The importance of NO in many kinds of vertebrate and invertebrate learning and memory is now appreciated (Hawkins 1996). NO inhibitors can block LTP in mammalian hippocampal brain slices as well as several forms of learning in a number of vertebrate and invertebrate animals (Schuman & Madison 1994; Hawkins 1996). Inhibition of NOS with N-G-monomethyl L-arginine in spontaneously hypertensive rats impairs spatial memory independent of alterations in systemic hemodynamics (Suzuki *et al.* 1996). Inhibition of NOS activity in the honeybee blocks long-term (24 h) memory from multiple training sessions but has no effect on initial learning or short-term (3 h or 8 h) memory (Muller 1996). NOS and its putative activity has been demonstrated histochemically in opisthobranch and cephalopod molluscs (Munoz *et al.* 1996; J. Bonaventura and J. D. Robertson, unpublished observations). We have shown that L-NAME blocks touch learning in *Octopus vulgaris*. (Robertson *et al.* 1994). In addition, we have shown that the L-NAME blockage of touch learning occurs with no observable loss of sensory or motor function (Robertson *et al.* 1995).

\* Deceased.

NO and related nitrogen oxides are toxic at high concentrations. The inducible form of NOS synthesizes the principle killing agent produced by macrophages. However, in nanomolar doses, it acts as a neurotransmitter and has several important regulatory and signalling functions in the nervous system. It stimulates guanylate cyclase leading to increased neurotransmitter release (Zhuo 1994; see also Montague *et al.* 1994; Meffert *et al.* 1994). The mechanism(s) by which NOS inhibition blocks learning and memory is unknown but one of its effects in low nanomolar concentration is stimulation of filopodial extension in growth cones (Wayne *et al.* 1993), even though at higher concentration ( $\mu\text{M}$ ) it causes growth cone collapse (Hess *et al.* 1994). We have discussed why we regard this function as particularly important in previous papers (Robertson *et al.* 1994, 1995). We have independent evidence from electron micrographic morphometric studies that filopodia are produced in large numbers in the neuropils in the octopus brain during touch learning (Robertson & Lee 1990) and have found that cytochalasins B and D, agents known to cause collapse of filopodia *in vitro*, prevent touch learning (Robertson & Lee 1990; Robertson 1994).

Recently it has been reported that the gene for brain NOS (nNOS) has been genetically deleted in mice and that subsequent application of NOS inhibitors still blocked LTP (O'Dell *et al.* 1994). These results were equivocal because endothelial NOS (eNOS) is also present in nerve cells and takes over the function of nNOS. Presumably a similar situation obtains in other organisms; hence, approaches that are complimentary to genetic deletions will be necessary to sort out the role of NO in learning and memory.

## 2. MATERIALS AND METHODS

Our general methods of handling Octopuses and using them to study learning and memory have been described in detail in recent papers (Robertson 1994; Robertson *et al.* 1994*b, c*) and we shall therefore abbreviate the description and concentrate here on the aspects peculiar to studying visual learning.

### (a) *Animal care*

Specimens of *Octopus vulgaris* weighing up to 1 kg were kept in Plexiglas tanks with a constant supply of sand-filtered, thermally controlled sea water, recirculated from reservoir tanks containing bacterial cultures for control of ammonia and nitrites. The water was partly replenished at intervals to control nitrates and routinely circulated through ultraviolet light sterilizing tubes. The octopus holding facility is well-established. The animals are kept with utmost care for their well-being, giving them as close to a normal living environment as possible. All of the individuals having contact with octopus were appraised of the great importance of treating the animals with great care and consideration.

### (b) *Phase I: preconditioning*

Each new animal was positively preconditioned by presentation of a smooth plastic ball  $\sim 2.5$  cm. in diameter with a daily feeding of shrimp. A black or white ball was dangled in the visual field *ca.* 10–20 cm away from the reach

of the arms on a clear plastic fishing line and moved up and down at  $\sim 1$  Hz. The animals can visually distinguish between black and white balls in our tanks (Robertson & Young 1988; Robertson & Lee 1990), but showed no apparent innate preference for either ball. The animal soon began to reach out and seize the ball and move it to its mouth. The ball was then immediately withdrawn and replaced by a morsel of shrimp. Soon the animal responded immediately on seeing the ball, jet-propelled toward it and seized it. If the animal did not react within 15 s, the ball was touched to an arm and it was then taken. Any animal consistently refusing the balls or food was discarded. After a few trials, training was begun on the group of animal that seized the ball as soon as it was presented. These animals showed no preference for the black or white ball during this preconditioning period.

### (c) *Phase II: training*

Training was done in twice daily sessions in which four smooth black plastic balls, and four similar smooth white balls were presented in pseudo-random order at *ca.* 5 min intervals. At first all animals responded positively to all eight balls. For group I, each animal was given a morsel of shrimp when it took the positive black ball; for taking the negative white ball it received a 5–6 V AC electrical stimulation. For group II, each animal was given a morsel of shrimp when it took the positive white ball; for taking the negative black ball it received a 5–6 V AC electrical stimulation. The food and shocking electrodes were always kept out of sight of the animal until the response had been given. The normal control animals soon responded to the positive ball by seizing it and ignored or backed away from the negative ball.

### (d) *Experimental NOS inhibition*

Experimental animals in this group received an intramuscular injection of L-NAME or D-NAME (the NOS-inactive enantiomer), 1 h before each training session. L-NAME or D-NAME was dissolved in Millipore 0.2  $\mu\text{M}$  filtered sea water in amounts calculated according to its weight, to give a brain concentration of 75  $\mu\text{g kg}^{-1}$ . This dose was chosen because it blocks LTP in mammalian brains (Schuman & Madison 1994) and was found effective in blocking touch learning in our previous work (Robertson *et al.* 1994; Robertson *et al.* 1995). We have so far done no dose-response studies. The solutions were sealed in vials coded by another investigator and frozen for storage before use. The experiments were all done blind.

### (e) *Data*

Data from each training session was plotted as bar graphs of cumulative positive and negative responses and as a learning index '*S*' where  $S = (X + N - Y) / 2N$ ; *X* = number of positive responses to the positive balls, *Y* = number of positive responses to the negative ball; *S* = 1 means perfect performance; *S* = 0.5 means no learning; *S* = 0 means 100% mistakes. We also plotted per cent correct responses, obtaining curves very similar to the *S* index curves but preferred the latter because it compensated for distortions sometimes introduced when only one or no balls were taken by one or more animals in a training session. Standard errors were calculated using the DeltaGraph Pro 3.5 Program for Macintosh (DeltaPoint, Inc., Harris Court, Suite B-1, Monterey, California 93940, U.S.A.). A total of two sample *t*-tests were used to calculate *t* and *p* statistics for each group of animals.

3. RESULTS

Figure 1 is a bar graph plot of the cumulative positive responses to the positive black balls and the negative white balls by the first set of four control animals injected with D-NAME and four test animals injected with L-NAME. Note that the animals all took the positive black ball consistently from the beginning to the end of the experiment but that they quickly stopped taking the negative white ball after the first few trials. The resulting curves from the D-NAME animals ( $n = 4$ ) in figure 1 differed significantly ( $t = 12.84$ ;  $p < 10^{-6}$ ). By contrast the corresponding curves from the L-NAME injected animals in figure 2 ( $n = 4$ ) showed no significant difference in the takes of the two balls throughout the experiment ( $t = 0.25$ ;  $p = 0.8$ ).

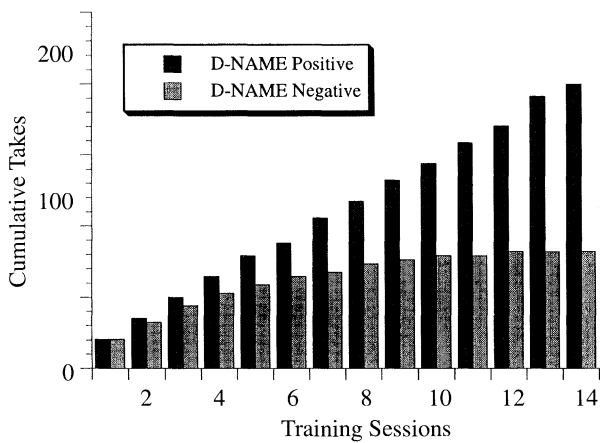


Figure 1. Bar graph of responses of D-NAME injected Octopuses in a visual learning paradigm. The solid black bars show the cumulative positive responses to the positive black balls and to negative white balls (hatched bars) in set 1 of four D-NAME injected control animals trained to the black ball positive paradigm. Note that the control animals stopped taking the negative balls after about 10–11 trials (two tailed  $t$ -test;  $n = 4$ ;  $t = 12.84$ ;  $p < 10^{-6}$ ). The number of trials required to learn the paradigm is the number usually needed in such experiments.

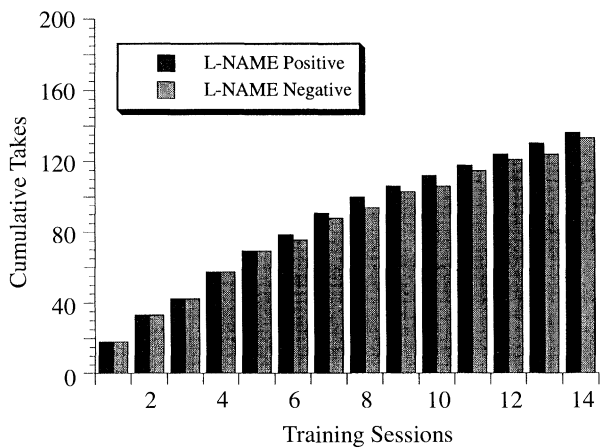


Figure 2. The corresponding graphs from the L-NAME injected animals in set 1, showing no significant difference in the takes of the black and white balls throughout the experiment hence indicating failure to learn the paradigm ( $n = 4$ ;  $t = 0.25$ ;  $p = 0.8$ ).

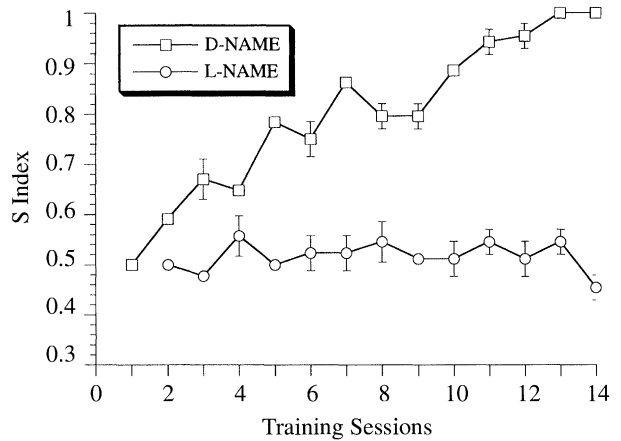


Figure 3. Line graph showing the learning index values  $S$  of the D-NAME and L-NAME injected animals. The error bars were calculated using the DeltaGraph Pro 3.5 Program for Macintosh (DeltaPoint, Inc., Harris Court, Suite B-1, Monterey, California 93940, U.S.A.). It is clear that the D-NAME injected animals learned the paradigm while the L-NAME injected ones did not.

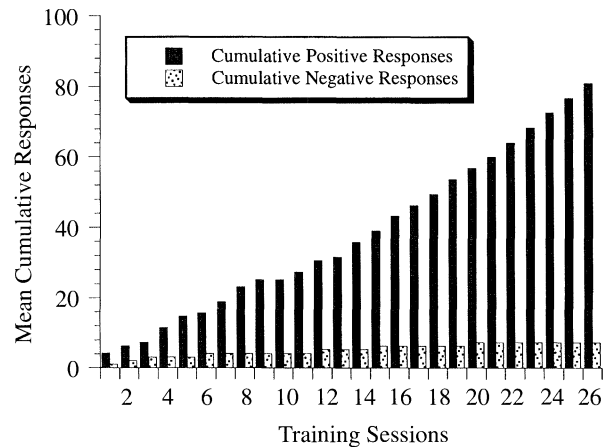


Figure 4. Data showing learning by the second set of animals, trained by another person at a later date but with the paradigm of the smooth white ball positive and black ball negative. This experiment was done in the fall of 1994 when the animals were much larger and somewhat sluggish in their responses. As usual with older animals, they learned to reject the negative ball with somewhat less speed and precision, although they had probably effectively learned the paradigm by about the 13th to 14th training session. The experiment in this case was run for a longer time with 25 training sessions but with only six animals. However, the animals learned the paradigm very well with no significant difference from the other set ( $n = 3$ ;  $t = 20.01$ ;  $p < 10^{-6}$ ).

Figure 3 is a plot of the  $S$  learning index values for this first set of animals trained to the black ball positive. Note that the D-NAME injected control animals reached  $S$  values of 1 after 13 training sessions, whereas the L-NAME injected animals continued to fluctuate around the chance value of 0.5 throughout. Clearly the control animals learned the paradigm while the test animals did not.

Figures 4 and 5 present bar graphs of data from a second set of experiments which was done with a separate set of animals by another trainer. The opposite

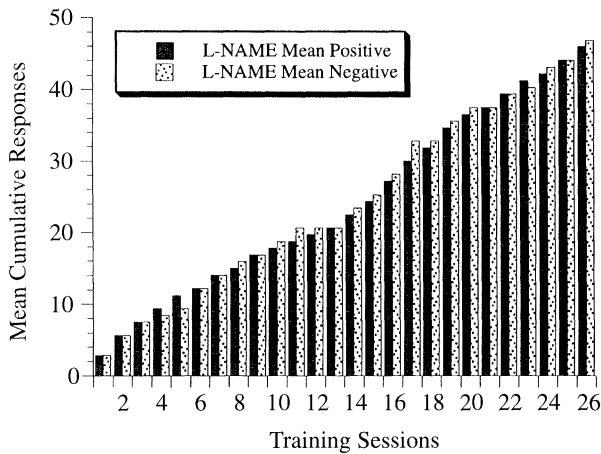


Figure 5. Bar graph showing that the L-NAME injected animals in the second set did not learn the paradigm despite the much larger number of training sessions ( $n = 3$ ;  $t = -0.13$ ;  $p = 0.9$ ).

paradigm, with the white ball positive, was used. The procedures used were the same, but the experiment was run for a longer time (26 training sessions) and six animals, three each for control and test. Whereas the net results were the same, slightly more variation in the responses was seen. The D-NAME injected controls (see figure 4) learned the paradigm ( $t = 20.01$ ;  $p < 10^{-6}$ ) but the L-NAME ones in figure 5 did not ( $t = -0.13$ ;  $p = 0.9$ ).

#### 4. DISCUSSION

These experiments complement and extend to another learning modality our previous work in which we showed that inhibition of NOS by injections of L-NAME blocked touch learning in *Octopus*. This is consistent with the fact that NOS inhibition negatively affects several different sensory modalities of learning in a variety of different animals. This suggests that the same general principles that apply to touch learning also operate in visual learning in *Octopus*. It also suggests that a mechanism involving NOS may well be associated with learning in all brains.

Visual learning may be more complex in *Octopus* than touch learning in that a much larger proportion of the brain mass is believed to be involved. The two optic lobes and the entire superior frontal system together constitute a considerably larger mass of neuronal tissue than the parts of the brain, mainly the inferior frontal lobe system, concerned primarily with touch learning. The vertical lobe, which is very important in visual learning is much larger than the subfrontal lobe, which seems to play a corresponding role in touch learning. It is believed that the inferior frontal system also participates in visual learning and vice versa (Young 1991). However, our recent work with cytochalasins (Robertson 1994) as well as earlier work by others (Young 1971; Wells 1978) has indicated that this is not an essential one.

Our past work with touch learning has resulted in several new findings that may be applicable to organismal learning in general and to our proposal that an important component to the process includes

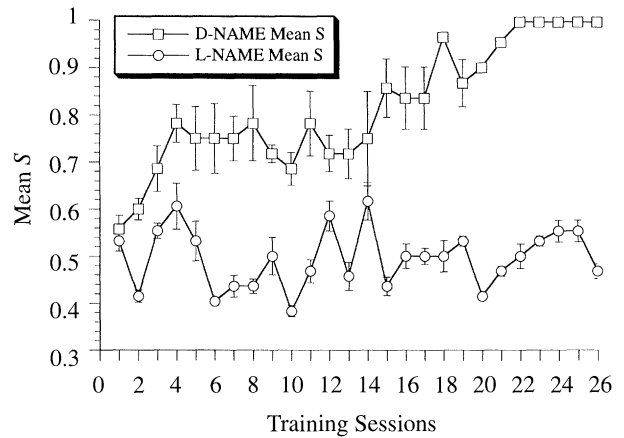


Figure 6. This line graph of the *S* Index values for the second set of animals shows clearly that the D-NAME injected animals learned the paradigm, while the L-NAME injected ones did not.

anatomical growth and fixation. The present results extend our findings to a different modality of learning in *Octopus*, adding some further reason to believe that there may be some general validity in these ideas. The general points are as follows.

1. We have shown by direct electron microscopic study of combined serial sections and morphometric analyses of filopodia in the subfrontal lobe of octopus, known to be importantly involved in touch learning (Young 1971; Wells 1978), that there is a significant increase in numbers of filopodia during learning of a touch paradigm (Robertson & Lee 1990).

2. NO, at nanomolar concentrations, has been shown to stimulate active and rapid extension of filopodia when directly applied in the vicinity of growth cones *in vitro* (Wayne *et al.* 1993).

3. Inhibition of filopodial extension either by direct action of cytochalasins or by limitation of NO blocks touch learning. On the basis of these facts we have theorized that extension of filopodia is an essential element of the learning process perhaps contributing significantly to changes in the efficacy of synaptic transmission.

There is increasing evidence that NO is involved in some of the fundamental aspects of the multiple and overlapping learning and memory systems (Hawkins 1996). Our studies with *Octopus* and the studies with honeybees (Müller 1996) suggests that NOS is involved with tactile, visual and olfactory learning and long-term memory in the invertebrates. Much of the previous work with vertebrates in which inhibition of NOS has resulted in deficits in learning, LTP and LTD has been questioned (Bannerman *et al.* 1994*a, b*). It is not yet clear whether or not these concerns are valid. It is relevant to note that the vertebrate findings are more difficult to interpret than the experiments we have reported in that the paradigms used are ones which are more complex than those we used in our octopus work. It is also important that probabilities calculated from *t*-tests usually fall in the  $p = 0.05$  range whereas our  $p$  values generally fall in the range of  $p < 10^{-6}$ . Further work is needed to settle these questions, but we believe our data is statistically more

valid than that derived from other sources. Our data is derived from much simpler procedures and is very consistent. We do not believe the objections raised by Bannerman *et al.* apply to *Octopus*.

The present results open new experimental approaches. Where is the primary action in visual learning? Is it in the vertical lobes, optic lobes, superior frontal lobe, or even the eye? Both the inferior and superior frontal systems, which are essential for touch and visual learning respectively, are characterized by the presence of enormous numbers of amacrine cells. Young has long postulated them to be involved in some crucial way in touch and visual learning. This is exemplified in his theory of learning (Young 1991). We are now in a position to isolate these cells along with the large motor cells in both the touch and visual systems and study their interactions *in vitro*, particularly with regard to formation of synapses and responses to endogenous as well as exogenous neurotransmitters, cytochalasins and NO. The new findings we report here open up investigative approaches to understanding *Octopus* learning and memory *in vitro* as well as in the living animal.

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