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Sex difference in rabbit eyelid conditioning

Monika Orłowska-Majdak, Paweł Kołodziejski and **Władysław Z. Traczyk**

Department of Experimental and Clinical Physiology, Institute of Physiology and Biochemistry, Medical University of Lodz, 6/8 Mazowiecka St.,
92-215 Lodz, Poland

Abstract. The rabbit eyelid conditioned reflex has been used to compare associative learning in males and ovariectomized females. A new method for monitoring eyelid movements is described. Rabbits were trained on simple delay classical conditioning. Conditioned responses were recorded during 8 acquisition days and 6 days of extinction training. Analysis of variance (ANOVA) and the least significant difference (LSD) post hoc test was used to analyze the data. The results showed that males achieved significantly better learning than females during the first day of acquisition but later they slowly attained the best result, contrary to females. Moreover, extinction of the conditioned reflex was significantly faster in females than in males. It is postulated that females learn and extinguish faster than males because of a higher level of brain plasticity.

Correspondence should be addressed to: Monika Orłowska - Majdak, Email: monikamajdak@poczta.onet.pl

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It is well known that mammals exhibit sex differences in certain types of learning abilities. There are some reports of sex differences in active avoidance learning (Van Oyen et al. 1981), appetitive learning (Shultz 1976) and a water maze task (Fry 1995) in rats, and in repeated T-maze learning (Lindzey and Winston 1962) and appetitive learning (Mishima et al. 1986) in mice. Females outperform males on classical conditioning eyeblink tasks and moreover exposure to an acute stressor had diametrically opposite effects on the rate of acquisition of this conditioned response in male vs. female rats (Wood and Shors 1998). No sex differences in radial maze performance (Kobayashi et al. 1988) and in the Morris water maze (Bucci et al. 1995) was observed in young rats. Aged male rats demonstrated superior radial maze performance compared to females (Kobayashi et al. 1988). It has been suggested that the male advantage in spatial tasks frequently documented in laboratory rodents and humans is due to age and a different course of maturation of the hippocampus in males and females (Sherry et al. 1992, Bucci et al. 1995, Lee et al. 1998).

It has been demonstrated that neuroanatomical sex differences are not absolute but can vary with the environment (Juraska 1984). Effects of inescapable shocks on subsequent behavior in an elevated plus-maze and on shuttlebox escape performance are different in male and female rats (Steenbergen et al. 1990). This experiment indicates that females' behavior seems to be less dependent on previous experience and more readily controlled by available contingencies. Males' behavior, on the other hand, is strongly controlled by previous experience, which is why they may be more inhibited in learning new contingencies (Steenbergen et al. 1990).

There is no information on sex differences in learning abilities in rabbits. We have used the classically conditioned eyelid response to study associative learning in male and female rabbits. To our knowledge all of the eyelid conditioning studies in rabbits used males as subjects. The effects of sex on classical eyelid conditioning in rabbits have not yet been examined.

Female rabbits underwent bilateral ovariectomy as the initial surgical intervention to prevent the possibility of reflex ovulation. It is well known that many external stimuli associated with mating, acting through the eyes, nose, ears, tactile and pressure receptors can influence reflex gonadotrophic hormone release bringing about ovulation (Harris and Campbell 1966). This might be an additional factor modifying learning abilities of the female rabbits. In rats the phase of the estrous cycle is associated with differential performance on the Morris water maze test of spatial memory: estrous females performed more poorly than diestrus and ovariectomized females (Frye 1995). In contrast, removal of circulating ovarian hormones partially impaired the rate of acquisition in rat's eyelid conditioning (Wood and Shors 1998). Ovariectomized females are more hormonally stable than intact ones.

Five male and six female adult New Zealand white albino rabbits, weighing above 3 kg, were housed conventionally (lights on 06.00 h and off 20.00 h) and given water and regular food *ad libitum*. Bilateral ovariectomy was performed under general anaesthesia. Female rabbits were premedicated with a subcutaneous injection of atropine sulphate (1.0 mg per animal) and anaesthetized with intravenous pentobarbital (Vetbutal, Biowet--Pulawy, 30 mg/kg). After immobilization the skin on the rabbit's abdomen, muscles and peritoneum were cut in the midline. To prevent the rabbit's bleeding, the mesovarium was ligated and then the ovaries were excised.

All experiments were carried out in accordance with the NIH guide for the care and use of the laboratory animals.

Rabbits were stereotaxically implanted with a plexiglass headpiece with guide cannulae. This surgical procedure has been described in detail in another paper (Traczyk et al. 1997). In brief, in animals under general anaesthesia (see above) five holes were made with a dental drill in the skull in places determined using the rabbit stereotaxic atlas (Sawyer et al. 1954). Four small holes were drilled for the guide cannulae leading to appropriate brain structures and the fifth large hole around the bregma for the 3rd ventricle cannula. After the lowering of the whole headpiece, the tip of the 3rd cerebral ventricle cannula was positioned 10 mm deep from the surface of the dura mater and the tips of the guide cannulae close below the dura mater. The tip of the 3rd cerebral ventricle cannula was used as a reference point. The headpiece was fastened to rabbit's skull bones with dental cement (Duracril, Spofa).

After surgery each rabbit received intramuscular injections of 100,000 IU of benzylpenicillin potassium (Polfa-Tarchomin) and 0.5 g of streptomycin (Polfa--Tarchomin) daily for five consecutive days. The stereotaxically implanted headpiece with the stainless steel guide cannulae for microdialysis probes was made of plexiglass. Its dimensions were: 21 mm x 18 mm x 25 mm. In these experiments the headpiece was used only to fix photoelectric transducer and an air puff nozzle on the rabbit's head. The metallic holder for photoelectric transducer and air puff nozzle was screwed into the back surface of the headpiece.

Following at least one month of postoperative recovery each rabbit was adapted to restraint in an attenuation chamber for approximately half an hour daily. Eyelid reflex conditioning began one week later. Rabbits were trained on simple delay classical conditioning. This Pavlovian paradigm involves the pairing of a tone (1 kHz, 70 dB, 450 ms) as the conditioned stimulus (CS) with a co-terminating air puff (0.2 kg/cm^2, 100 ms) directed at the left eye as the unconditioned stimulus (US) on each trial. The training consisted of 120 consecutive trials per day. The intertrial interval averaged 22 s. The rabbits learned to a criterion of 80% conditioned responses and were overtrained to 8 days. After the acquisition training rabbits underwent 6 day of extinction. During the extinction session only 120 conditioned stimuli (tones) were applied. Such pattern of training was repeated three times, i.e., three 8 day acquisition periods and three 6 day extinction periods (i.e. three courses).

A special system was built for delivery and control of sensory stimuli and for the control of intertrial intervals as well as for detection of the eyeblink responses. The system has been described in detail in a separate communication (Orłowska-Majdak et al. in press). A phonopneumatic stimulator generated periodic air puffs and tones and controlled the recorder. A photoelectric transducer using close infrared converted movements of the eyelid to electric signals. The photoelectric transducer was mounted together with the air puff nozzle with a special holder and the screw on the rabbit's headpiece, about 2 cm from the cornea. Such instrumentation did not restrict the head rotation. Eyeblinks were detected by changes in the reflection of light from the cornea or eyelid. The degree of light reflection was proportional to the degree of closure of the eyelid. When the eye was closed, maximum light was reflected and the maximal response amplitude was observed.

Responses occurring during acquisition in the CS-US interval, with amplitude exceeding 1 mm, were classified as conditioned responses (CRs). Responses elicited during extinction were classified as CRs if they were initiated during the CS and their amplitudes exceeded 1 mm. Conditioned responses were calculated as a percentage of all 120 conditioned stimuli applied during one day session. Fig. 1 illustrates examples of eyelid conditioning recording during acquisition and during extinction.

All 11 rabbits adapted easily to the experimental procedure. Percentage of conditioned responses were subjected to an analysis of variance (ANOVA) involving the factors of sex, course and day with repeated measures on the last factor, separately for acquisition and extinction training. For statistical analysis the percentage were transformed to arcsine values according to the formula: $2 \times \arcsin\sqrt{p}$. Analysis of the acquisition data yielded significant effects of day ($F_{7,189}$ = 27.6, P=0.000). The sex and course by day interaction was not significant ($F_{14,189}$ = 0.9, P=0.528). On the other hand, the sex by day interaction was significant ($F_{7,189}$ = 3.0, P=0.005) as was the course by day interaction ($F_{14,189}$ = 3.2, P=0.000). The least significant difference (LSD) post hoc test was used following a significant ANOVA. The LSD showed a significant difference between the percentage of conditioned responses achieved by males and females on the 1st day of training (P=0.001). The mean value for males was 48.7 ± 5.7 %. That for females was 32.0 ± 4.5 % (mean SE). Furthermore, males reached a higher percentage of responses during acquisition, from the 1st day up to the 4th day (P<0.05). Females reached the highest percentage of responses as early as the 2nd day of acquisition (P=0.000) (Fig. 2). Post hoc analysis of the course by day interaction showed that males achieved a higher level of acquisition than females on the 1st day in all 3 courses, but the difference was statistically significant only in the 3rd course (P=0.007). Analysis of the extinction data showed significant effects of sex ($F_{1,23}$ = 9.4, P=0.005) and day ($F_{5,115}$ = 73.2, P=0.000). The interactions of sex by day ($F_{5,115}$ = 2.8, P=0.02) and course by day ($F_{10,115}$ = 2.2, P=0.02) were also significant (P=0.02). Moreover, the 3 factor interaction (sex x course x day) approached significance ($F_{10,115}$ = 1.8, P=0.069). The LSD test showed that female data differed from male data on the 1st, 2nd and 3rd day of extinction (P=0.000). During extinction training the percentage of responses in males diminished more slowly than in females over the first 4 days. Females yielded the smallest percentage of responses on the 3rd day, and males on the 4th (P<0.05) (Fig. 2). The significant course by day interaction was analyzed by the post hoc LSD test. The male response rate was higher than that of female on most days during the 6 days of extinction in all 3 courses but significant differences were seen only on the 3rd day in the 2nd course and on the 6th day in the 3rd course.

Results of our study suggest that there exists a sex difference in the course of acquisition and extinction in eyelid conditioning in male and female rabbits. Males

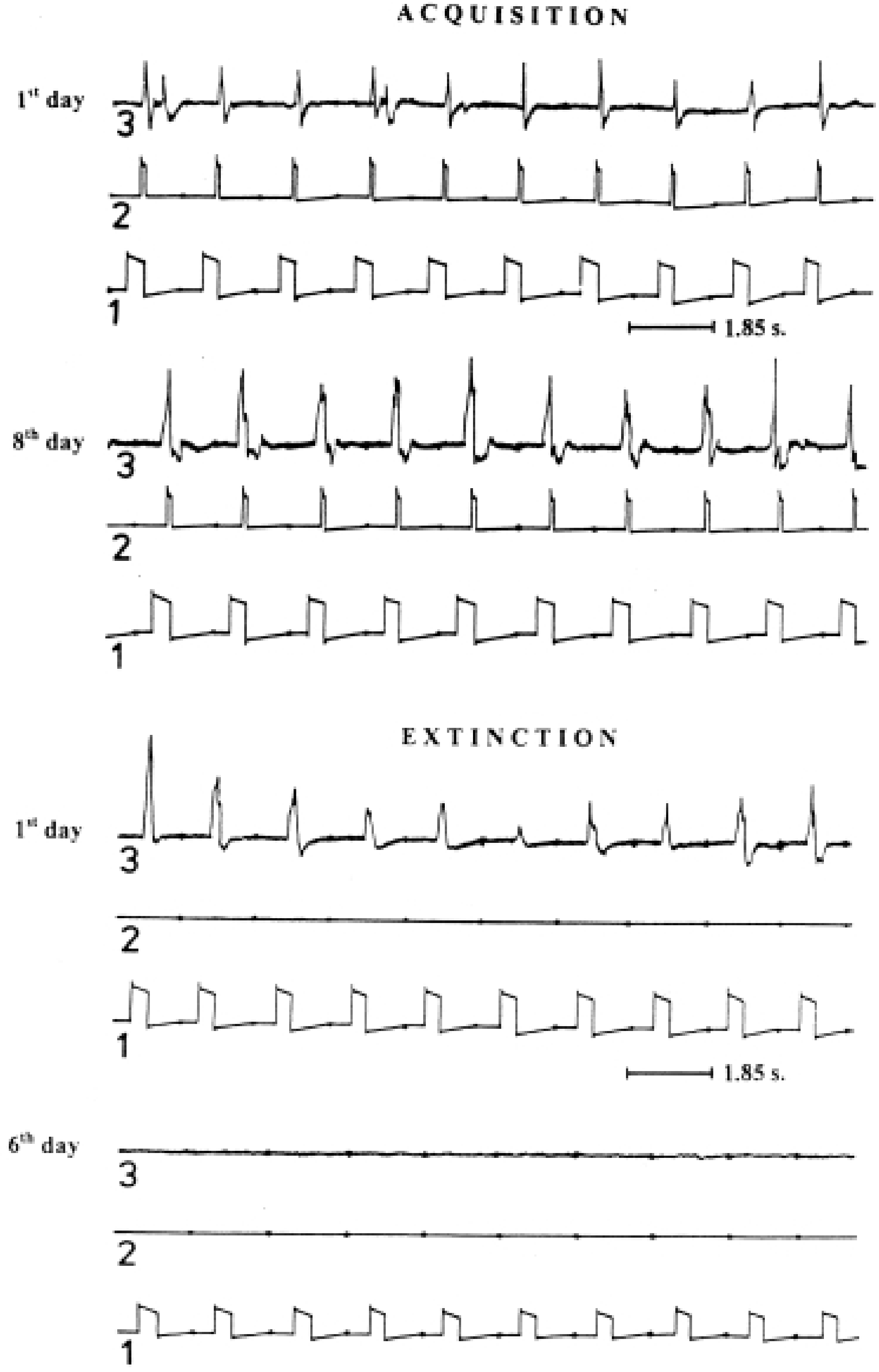

Fig. 1. An example of peristimulus eyelid responses from several trials of rabbit eyelid conditioning during acquisition and extinction: 1, tone (CS), 2, air puff (US), 3, eyelid responses (CRs). On the 1st day of acquisition eyelid movements began after the air puff onset. After acquisition training, on the 8th day, the eyelid movements were caused by the tone itself and began before the air puff onset. On the 1st day of extinction responses elicited by CS alone are visible. On the 6th day of extinction rabbits did not respond to CS.

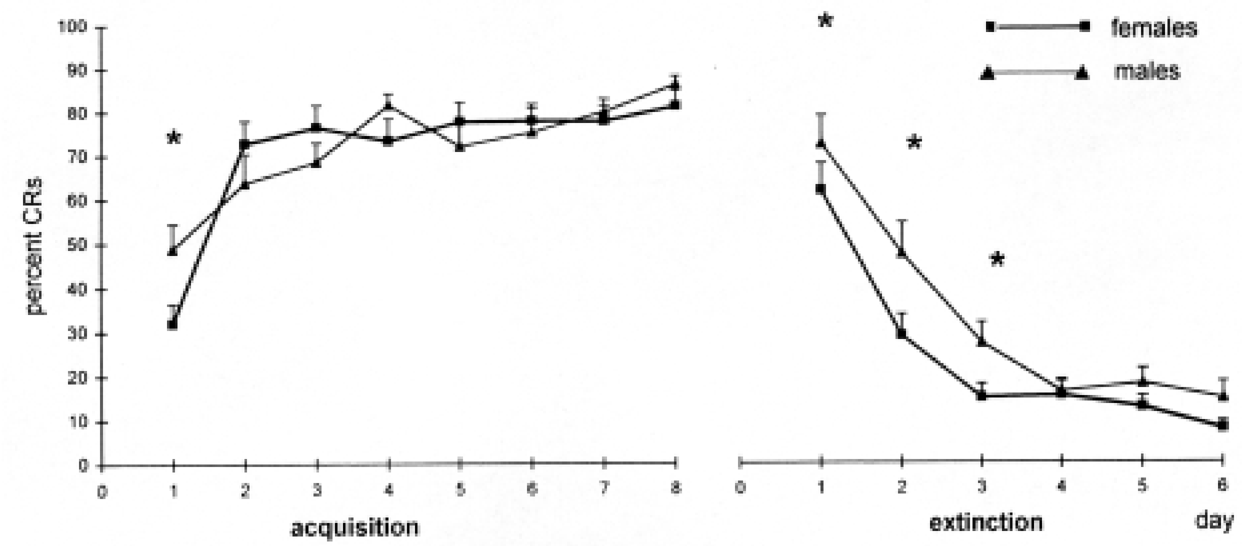

Fig. 2. A comparison of conditioned responses in males and females. Curves show the mean (SE) percentage of responses SE in a 120 trial block of acquisition and extinction (mean of 3 courses). Statistically significant differences in male and female responses were observed on the 1st day of acquisition (*P*=0.001) and on the 1st, 2nd and 3rd day of extinction (*P*=0.000) - see the asterisks.

achieved better learning than females on the 1st day of acquisition, but the best results they attained on the 4th day, contrary to females. Female rabbits attained their best results on the 2nd day of training. Moreover, extinction of the conditioned reflex progressed faster in females than in males. Female rabbits learned the eyelid reflex faster than male rabbits, but both sexes achieved the same final level of acquisition. The lowest value of conditioned responses during extinction appeared earlier in females than in males and the level of extinction was lower in females than in males during the first three days of training. The course of acquisition and extinction characteristic for each sex was observed in all 3 courses of training.

In recent work on sex differences in eyeblink response in the rat it was found that females acquired conditioned responses faster than males (Wood and Shors 1998). Our findings in rabbits are similar, but the difference is not as marked as in rats. Moreover, contrary to the results in rats, the percentage of responses during the 1st day of acquisition was higher in male rabbits than in females.

Neuroanatomical and hormonal differences reported in animals of both sexes may be responsible for the observed differences in behavior. Gonadal steroid hormones may influence CNS functioning through a variety of different mechanisms. Estradiol accelerates functional brain maturation by shortening the myelination period (Curry and Heim 1966). Neonatal testosterone treatment of female rats resulted in their developing a more male - like hippocampus. These treated females performed as well as males on a spatial navigation task (Roof and Havens 1992). The development and androgenic regulation of sex differences were described using motoneuron number within a sexually dimorphic rat spinal nucleus (Nordeen et al. 1985). Recently, it was shown that estradiol enhanced hippocampal synaptic plasticity by an increase in spine density in pyramidal neurons (Pozzo-Miller et al. 1999). Progesterone prevents this effect (Murphy and Segal 2000). Moreover, estrogen enhanced N-methyl-D-aspartate (NMDA) receptor - mediated currents and promoted an enhancement of long term potentiation (LTP) magnitude (Foy et al. 1999). In our experiments rabbits were ovariectomized before the conditioning training. Therefore, only the perinatal activity of the gonadal sex hormones can be responsible for higher level of brain plasticity in female rabbits and gender related differences in behavior.

This is the first report that eyelid reflex conditioning is gender related in rabbits.

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