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STEREOLOGICAL ESTIMATION OF PURKINJE NEURON NUMBER IN C57BL/6 MICE AND ITS RELATION TO ASSOCIATIVE LEARNING

D. S. WOODRUFF-PAK[a,b]*

[a]*Department of Psychology, Temple University, 1701 North 13th Street, Philadelphia, PA 19122, USA*

[b]*Albert Einstein Healthcare Network, Philadelphia, PA 19141, USA*

Abstract—Cerebellar Purkinje neurons are among the most vulnerable neurons in the CNS. Impairment in Purkinje neurons has consequences for cerebellar cortical-dependent forms of behavior. The primary aim of this study was to evaluate Purkinje neuron number over the lifespan of C57BL/6 mice. Stereological estimates of the total number of Purkinje neurons in cerebellar cortex were made in 25 C57BL/6 mice aged 4, 8, 12, 18, and 24 months. Delay eyeblink classical conditioning to a white noise conditioned stimulus was also assessed for 10 daily sessions. Statistically significant age differences in Purkinje neuron number were observed beginning at 18 months. Delay eyeblink conditioning also showed significant age-related impairment, at least some of which resulted from age-related deficits in hearing. Eliminating the hearing-impaired 18- and 24-month-old mice from the analysis, the correlation between Purkinje neuron number and rate of conditioning was −0.435 (P= 0.053) in 15 younger mice aged 4–12 months. Purkinje neurons are one of the few types of neurons showing significant age-associated loss. Results indicate that individual variation in Purkinje neuron number is related to eyeblink conditioning in young organisms suggesting that reserves of neuron numbers against which individuals draw are defined early in life.

Key words: cerebellum, optical fractionator, normal aging, delay eyeblink classical conditioning, age-related hearing loss.

Purkinje neurons are among the few neurons in the CNS exhibiting significant loss with normal aging (Andersen et al., 2003; Larsen et al., 2000). Unbiased stereological techniques indicate that the magnitude of cortical neuron loss once attributed to normal aging was overestimated (West et al., 1994; Pakkenberg and Gundersen, 1997). However, there is significant age-related Purkinje neuron loss in the cerebellar cortex. In a stereological study of the entire cerebellar cortex, there were 11% fewer Purkinje neurons in 23-month-old Sprague–Dawley rats (Larsen et al., 2000). In 26-month-old rats, many Purkinje neurons appeared defoliated, with small distal dendrites and spiny branchlets being the most affected (Rogers et al., 1981). Related to the Purkinje neuron loss in these rats was a loss in synaptic density (Rogers et al., 1984), and electrophysiological studies identified a number of neuron firing parameters that were affected (Rogers et al., 1980). In particular, increasing numbers of aberrant, very slow-firing neurons were encountered in older animals. Age-related dysfunction of the cerebellar β-adrenergic receptor was observed to affect spontaneous firing as well as modulating the effects of other neurotransmitters such as GABA (Bickford et al., 1985). This age-related decline in cerebellar β-adrenergic receptor function has been postulated to underlie, in part, age-related deficits in motor learning (Bickford, 1995; Bickford et al., 2001; Gould and Bickford, 1996).

Purkinje neuron counts over the entire cerebellum of various age groups of C57BL/6 mice indicated significant loss after the age of 18 months (Hadj-Sahraoui et al., 1997; Doulazmi et al., 1999). Dendrites of Purkinje neurons also showed morphological changes in old C57BL/6 mice (Hadj-Sahraoui et al., 2001). In 4-month-old mice, dendrites of Purkinje neurons were richly branched, and heavily spined dendrites filled the molecular layer above the soma. In 22-month-old mice Purkinje neuron dendrites had clearly shrunk. One structural reflection of the diminished dendrites was that the thickness of the molecular layer at 22 months of age (146±5 μm) was reduced when compared with molecular layer thickness in 4-month-old mice (181±5 μm). At 12 months of age the thickness of the molecular layer was already significantly reduced.

Purkinje neurons in cerebellar cortex are normally engaged in a form of associative learning called eyeblink classical conditioning (Berthier and Moore, 1986; Gould and Steinmetz, 1996; Green and Steinmetz, 2005; Schreurs et al., 1991). In rabbits, there is a high correlation between Purkinje neuron number and acquisition of conditioned responses (CRs) to a tone conditioned stimulus (CS) (Woodruff-Pak et al., 1990; Woodruff-Pak and Trojanowski, 1996). Eyeblink conditioning has been studied across a variety of mammalian species, and striking parallels have been demonstrated among species in behavior and neurobiological substrates. Humans begin to show age-associated deficits in eyeblink conditioning at about 40–50 years (Woodruff-Pak and Thompson, 1988; Solomon et al., 1989) with no further statistically significant deficits between the age decades of the 50s through the 80s (Woodruff-Pak and Jaeger, 1998). Like humans, rabbits begin to show age-associated deficits in eyeblink classical conditioning in middle age (Coffin and Woodruff-Pak, 1993; Solomon and Groccia-Ellison, 1996). These results with humans and rabbits generalize to other mammals including mice (Kishimoto et

*Correspondence to: D. S. Woodruff-Pak, Department of Psychology, 1701 North 13th Street, Philadelphia, PA 19122, USA. Tel: +1-215-204-1258; fax: +1-215-204-5539.
E-mail address: pak@temple.edu (D. S. Woodruff-Pak).

Abbreviations: ANOVA, analysis of variance; CR, conditioned response; CS, conditioned stimulus; DC, direct current; EMG, electromyography; HSD, honestly significant difference; PC, personal computer; s.d., standard deviation; SPL, sound pressure level; TC, trials to learning criterion; US, unconditioned stimulus.

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al., 2001; Vogel et al., 2002), rats (Knuttinen et al., 2001; Weiss and Thompson, 1991, 1992) and cats (Harrison and Buchwald, 1983). In all mammalian species that have been examined, the cerebellum is the essential site for acquisition and retention of the conditioned eyeblink response. Cerebellar cortical and nuclear regions ipsilateral to the conditioned eye are normally engaged in acquisition in delay eyeblink classical conditioning (McCormick and Thompson, 1984; Steinmetz, 1996; Thompson and Krupa, 1994), and the interpositus nucleus ipsilateral to the eye receiving the unconditioned stimulus is the essential cerebellar substrate (for a review, see Christian and Thompson, 2003).

The major aim of this study was to evaluate Purkinje neuron number over the lifespan of the mouse. Delay eyeblink classical conditioning to a white noise CS was also assessed in these mice. The C57BL/6 mouse strain was chosen as a commonly used mouse strain in research on aging and a strain on which most transgenic mice are built. A limitation of the C57BL/6 mouse strain for behavioral research is genetically based age-related hearing loss (Johnson et al., 2000; Willott, 1986). C57BL/6 mice in the 4–12 month range have relatively normal hearing and normal eyeblink conditioning to an auditory CS, but the conditioning data for C57BL/6 mice in the 18–24 month range are confounded by age-related hearing loss. Thus, we limited conclusions about Purkinje neuron and associative learning relationships to data collected in the first half of the mouse lifespan. We used unbiased stereological techniques (West, 1993) to count Purkinje neurons. To the best of our knowledge, this study represents the first report using unbiased stereological assessment of Purkinje neurons over the lifespan of C57BL/6 mice.

EXPERIMENTAL PROCEDURES

Animals

Unbiased estimates of cerebellar Purkinje neuron number were carried out in a total of 25 C57BL/6 mice previously tested in 500 ms delay eyeblink classical conditioning. Five mice were tested in each of the 4-, 8-, 12-, 18-, and 24-month age groups. There were two male and three female mice in all age groups with the exception of the 24-month group that had five female mice. Male and female C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and bred and aged in the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) -approved Central Animal Facility of Albert Einstein Healthcare Network. The colony room was temperature and humidity controlled and ventilated using a dedicated system. Room lighting was timed for a 12-h light/dark schedule. Mice were grouped by gender at weaning, with no more than 10 per cage, and housed individually beginning at least 5 days prior to testing. Housing consisted of a polycarbonate microisolator filtered-top cages. All mice had *ad libitum* access to sterile food and water. The Albert Einstein Healthcare Network's Institutional Animal Care and Use Committee (IACUC) reviewed and approved breeding practices and testing procedures. This research was carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The minimum number of animals required to show an effect was used, and procedures were designed to minimize or completely eliminate pain and distress.

Eyeblink classical conditioning

Surgery to implant recording and stimulating electrodes was carried out on all mice under anesthesia induced with isoflurane. The mice were covered with gauze strips to maintain normal thermoregulation during surgery. Four Teflon-coated stainless steel wires (A-M Systems, Inc., Everet, WA, USA), soldered to a four-pin male header (Jameco Electronics, Belmont, CA, USA), were implanted intramuscularly in the orbicularis oculi of the left upper eyelid. Wires were stripped of Teflon and carefully placed so that only the muscle-embedded wire was bare. The wires were glued onto the skull, and a four-pin headstage was cemented to two skull screws and the skull, and the incision closed. The two wires most rostral were used to record differential electromyography (EMG), and the two most caudal were used to deliver the electric shock. The duration of the surgery was about 45 min per mouse. Following surgery, mice were given Baytril antibiotic (85 mg/kg s.c.) to prevent infection and Buprenex anesthetic (0.075 mg/kg s.c.) for analgesia. A recovery period of 4–5 days was allowed before eyeblink conditioning sessions began.

The eyeblink classical conditioning apparatus was housed in four sound- and light-attenuating chambers (Med Associates, Inc., St. Albans, VT, USA). The tone was delivered through a wall-mounted speaker. Each chamber contained a round glass beaker in which the mouse was placed during testing. A copper Faraday cage enclosing the beaker was used as a shield to minimize external electromagnetic interference during recording. A shielded four-conductor wire entered the ceiling of the chamber and was used to deliver a direct current (DC) pulse (0.3 mA) to the orbicularis oculi and to record EMG. The EMG was passed through a 300–5000 Hz filter and amplified by 10,000. The signal was integrated and digitized before being read into an International Business Machines personal computer (PC) –compatible system. The white noise-burst was delivered by a Coulbourn Instruments tone/noise-generator (Model A69-20) with a built-in attenuator function. Each training session was run by the PC using software written in C++ language (Chen and Steinmetz, 1998). Data were collected in random access memory and saved to a hard drive for offline analyses.

A fan connected to the chambers created a background noise level of 70 dB sound pressure level (SPL). Background noise and CS intensity were calibrated on a weekly basis inside each of the four testing chambers with an Extech Instruments Sound Level Meter (Model 407735) with a frequency sensitivity of 31.5 Hz to 8 kHz. Each paired classical conditioning trial consisted of a 600 ms white noise burst (85-dB SPL, 10 Hz–20 kHz frequency range) CS that coterminated with a 100-ms 0.3 mA DC pulse unconditioned stimulus (US) generated by an Isostim™ Stimulator/Isolator. The inter-trial interval was 25 ± 5 s. Each block consisted of nine paired CS–US trials and one CS-only test trial. A total of 100 delay eyeblink classical conditioning trials were presented per session which were analyzed as nine blocks of 10 paired CS–US trials and one block of 10 CS-alone trials (Fig. 1). Mice were tested for 10 daily sessions.

Each session was computer-scored and also visually checked to confirm the accuracy of the automated scoring procedure. Whenever the EMG recorded from the orbicularis oculi exceeded 5 s.d. (standard deviations) above a baseline established in the 249 ms Pre-CS period, a response was considered to have occurred. A CR was scored if a response occurred after the 60 ms startle period and before the US onset (between 61 and 499 ms after CS onset). A short-latency or "alpha" response was scored if the response occurred within the first 60 ms after the CS onset. A bad trial was registered if the response amplitude exceeded 5 s.d. within the 100 ms prior to CS onset. Dependent measures reported for this study include percentage of CRs in the paired condition and trials to a learning criterion (TC) of eight CRs in nine consecutive trials in a session that contained a minimum of 40%