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Variation of Brain and Serum Cholinesterase Activity with Age in Wild Small Mammals

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The use of organophosphate (OP) and carbamate pesticides can result in exposure, and in some cases mortality, of non-target vertebrates such as small mammals (Westlake et al. 1980; Johnson *et al.* 1991; Hardy *et al.* 1993). These pesticides are anticholinesterases and inhibition of blood cholinesterase (ChE) activity is widely used as a biomarker to assess exposure in vertebrates (Thompson 1991); inhibition of brain acetylcholinesterase (AChE) activity by more than 40% is generally considered to be associated with impaired physiological function and altered behaviour (Grue *et al.* 1991). Determination of the degree to which ChE activity has been inhibited requires good base-line knowledge of normal (control) activity levels. While normal levels of ChE activity have been measured in a variety of wild species (Westlake *et al.* 1983), the data have been obtained from a relatively small number of individuals. The difficulties in defining control activity levels from limited data have been highlighted by a long-term study in which normal brain ChE activity in wild-trapped wood mice *Apodemus sylvaticus* was found to vary by up to 33% during a twelve year period (Greig-Smith 1991). Such large-scale variation makes it difficult to identify when pesticide-mediated inhibition of activity has occurred.

ChE activity can vary with a variety of physiological factors (Rattner and Fairbrother 1991). One such factor is age; studies on the laboratory rat have demonstrated that brain AChE activity increases threefold after birth and reaches levels similar to those observed in adults at about the time of weaning (Rattner and Fairbrother 1991). However, no studies on the effects of age on ChE activity have been reported for wild mammals. Furthermore, little is known generally for mammals about whether brain and serum activity changes as adult animals grow old or whether serum ChE activity varies with age at all. Age is difficult to assess in wildlife, especially small mammals, and thus it is important to determine whether age could be a significant and confounding factor when diagnosing if ChE activity is inhibited in free-living animals. Indeed, it has been argued that studies on the effects of increasing

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age on ChE activity in wildlife species are urgently needed (Rattner and Fairbrother 1991). The *aim* of the present study was to determine whether age affects normal brain and serum ChE activity in the wood mouse and the field vole *Microtus agrestis*, two species of wild small mammal which occur in and around cereal fields and orchards and so may be exposed to OP and carbamate pesticides.

MATERIALS AND METHODS

Brain and serum ChE activity were measured in 105 captive-bred wood mice and in 13 captive-bred field voles of known age. All animals were maintained indoors at 18°C and most were kept on a constant photoperiod (14L:10D) although some wood mice of all ages were maintained under natural daylength as part of another experiment; no differences in ChE activity were detected between animals of the same age maintained on different photoperiods. Animals were kept either in breeding pairs or family groups and food and water were given *ad libitum.*

Animals were sacrificed by cervical dislocation followed by decapitation; anaesthesia was not used because it is thought to interfere with ChE activity (Vernadakis and Routledge 1973). Trunk blood was collected, allowed to clot and separated by centrifugation (2000 rpm at 4°C for 10 minutes), serum subsequently being kept on ice until analysed. The brain of each animal was excised immediately after death, weighed and then homogenised in 25 mM Tris-HCl buffer (containing 0.1% Triton X-100) using a glass homogeniser; the concentration of the resultant homogenate was 0.06 g ml^{-1}. Aliquots of each homogenised brain were kept on ice until analysed. Cholinesterase assays were performed as soon as possible (always the same day) after the collection of serum and brain samples.

The ChE activity in brain and serum was measured by the calorimetric method of Ellman *et al.* (1961), as adapted by Johnston *et al.* (1989) except that 0.287 mM acetylthiocholine iodide was used as a substrate in the reaction cuvette in the present study. Activity was determined in 5 µl serum samples and 20 µl brain homogenate, the final assay volumes being 3.0 ml and 3.015 ml respectively. Absorbance was measured at 412 nm and 37°C using a Unicam SP 1800 Ultraviolet Dual Beam Spectrophotometer (Unicam Ltd, Cambridge, UK), the spectrophotometer being run in dual beam mode with blanks (no substrate) to ensure that there was no endogenous DTNB colour change in the reaction cuvette which was unrelated to the substrate. Tissue blanks were also run to confirm that there was no spurious activity in the assays. Each assay was run for 3-4 minutes to ensure that the linear phase of the reaction was measured and the mean value of five replicate assays was taken as the ChE activity in each sample. Replicate assays of a commercial reference material (Precinorm U, Boehringer Mannheim UK, Lewes,

E. Sussex, UK) were analysed before and after the unknowns to ensure that the assay gave consistent results. The activities detected in the reference material were always within the manufacturer's specified range.

The relationships between age and brain AChE activity were suggested by distance-weighted least squares smoothing and analysed by non-linear regression (Silverman 1986); the models fitted were highly significant and had higher R^2 values than those for a linear regression model. There was no evidence of a non-linear relationship between age and serum ChE activity and standard linear regression analysis was carried out, incorporating sex as a factor in the model if this term was statistically significant.

RESULTS AND DISCUSSION

The brain AChE activity of male and female wood mice increased two-three fold during the first few weeks of life, rose to an average maximum of 12-13 µmoles $min^{-1} g^{-1}$ when mice were 7-10 weeks old, and then appeared to decrease to a "steady state" level (Figure 1); this relationship, as fitted by a critical exponential curve, was an improvement (higher R^2) over one which simply rose to an asymptote. The curves defining the relationship between age and brain AChE activity in male and female wood mice were not parallel ($F_{3,97}$=3.94, P<0.05); compared with females, males had slightly higher maximum brain AChE activities which declined more slowly to a "steady state" level. The range between the maximum and minimum activities in all the wood mice was 11.8 µmoles $min^{-1} g^{-1}$ which was equivalent to 65% of the highest activity measured.

Brain AChE activity appeared to vary little with age in wood mice more than 20 weeks old. The mean (±SE) brain AChE activity for these older male and female mice was 10.5±0.7 and 11.7±0.3 µmoles $min^{-1} g^{-1}$ respectively and the difference between the sexes was not significant (student t-test, t_{81}=1.58, P>0.05). Although brain AChE activity can vary with environmental and physiological factors (Rattner and Fairbrother 1991 for review), the brain AChE activity in captive-bred wood mice did appear to be typical of that in free-living animals; the mean (±SE) brain AChE activity measured in mixed sex groups of 16 and 6 wood mice taken from the wild was 10.1±0.49 µmoles $min^{-1} g^{-1}$ (Westlake *et al.* 1983) and 11.0±1.03 µmoles $min^{-1} g^{-1}$ (Shore and Fishwick *unpub. data*) respectively, similar to the activities measured in the present study in captive animals.

Brain AChE activity in field voles (Figure 2) was measured only in a limited number of females over 9 weeks old. Hence, it was not possible either to define the relationship between age and brain AChE activity with great accuracy nor to determine whether juveniles had lower ChE activities than adults. However, brain AChE activity in field voles appeared to vary with age in a pattern similar to that in wood mice, activity increasing to a

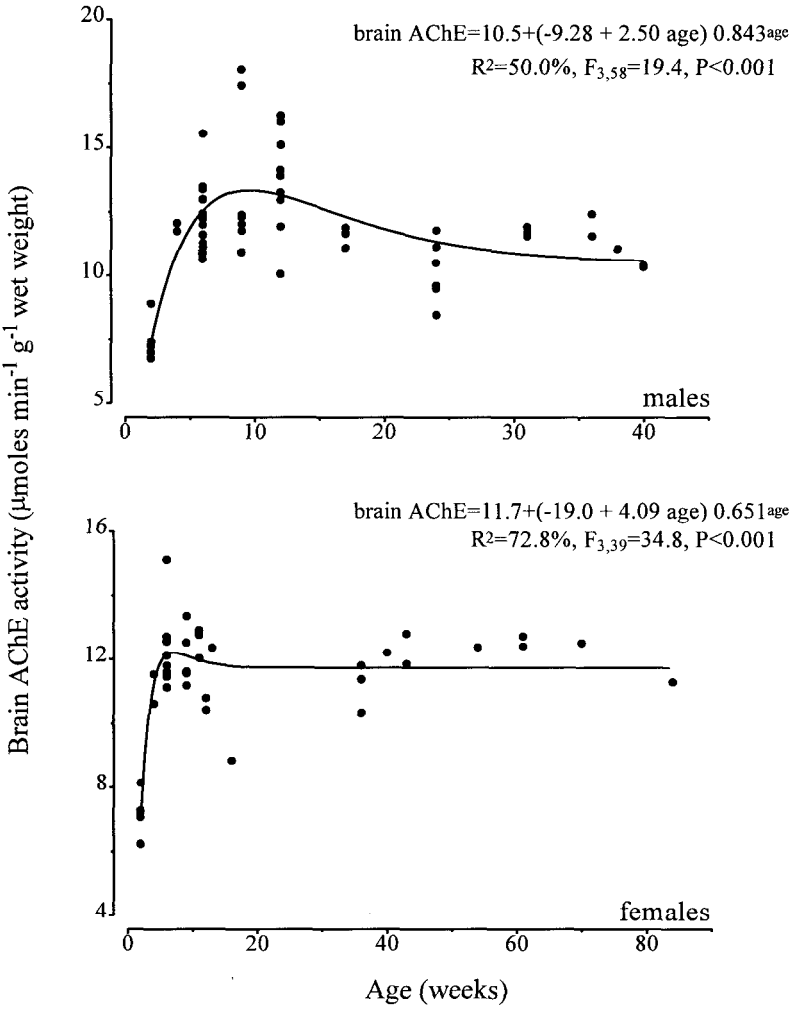

Figure 1. Variation in brain AChE activity with age in wood mice. The number of mice of the same sex and age in which AChE activity was measured varied between 1 and 17. Note that the scales of the graphs for males and females differ.

maximum and then decreasing to a "steady state" level (Figure 2); this relationship was an improvement (higher R^2) on one fitted by linear regression in which activity declined at a constant rate as age increased. In contrast to wood mice, peak brain AChE activity in field voles occurred at 20 rather than 7-10 weeks of age and a "steady state" activity only occurred when animals were approximately 80 weeks old; relatively few animals live this long in the wild. As in wood mice, there was a large range between the maximum and minimum brain AChE activities in field voles (50% of the

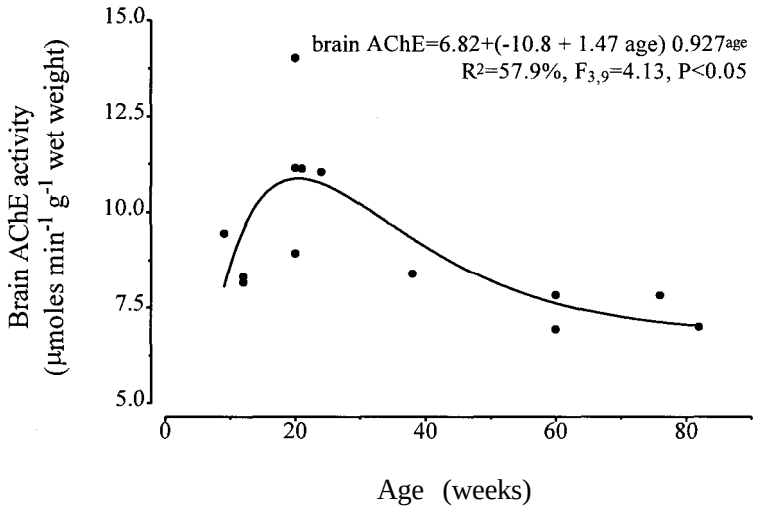

Figure 2. Variation in brain AChE activity with age in female field voles.

highest activity) and activity in captive-bred voles was typical of that in free-living animals; the mean (±SE) brain AChE activity in a mixed sex group of 10 wild-trapped field voles was 11.23±0.44 µmoles $min^{-1} g^{-1}$ (Westlake *et al.* 1983) which was within the range of activities measured in captive-bred animals in the present study.

Serum ChE activity appeared to vary little with age. In wood mice, sex was not a significant factor in explaining the variation in serum activity (t_{96}=1.02, P>0.05) and the relationship between ChE activity and age was subsequently analysed for both sexes combined (Figure 3). Although there was a statistically significant positive relationship, the rate of increase in activity per week was only 0.007 µmoles $min^{-1} ml^{-1}$ and age explained very little of the variation in the data (Figure 3); thus, a difference in age between groups of wood mice is unlikely to bias any comparison of their serum ChE activity. The mean (±SE) serum ChE activity of both male and female mice combined was 2.04±0.05 µmoles $min^{-1} ml^{-1}$ (n=93). In female field voles, there was no significant relationship between age and serum ChE activity (Figure 3) although the number of animals analysed was small. The mean (*SE) serum ChE activity in female voles was 2.83±0.25 µmoles $min^{-1} ml^{-1}$ (n=12) and was significantly greater (student t-test, t_{12}=2.76, P<0.02) than that in female wood mice (2.12±0.06 µmoles $min^{-1} ml^{-1}$, n=41).

The results of the present study demonstrate that brain AChE activity varies markedly with age in wild rodents. Wood mice appear to be similar to laboratory rats in that brain esterase activity in juveniles is much lower than that in adults. However, provided that the relationship between brain esterase activity and age is known, detection of low brain AChE activity in wild-

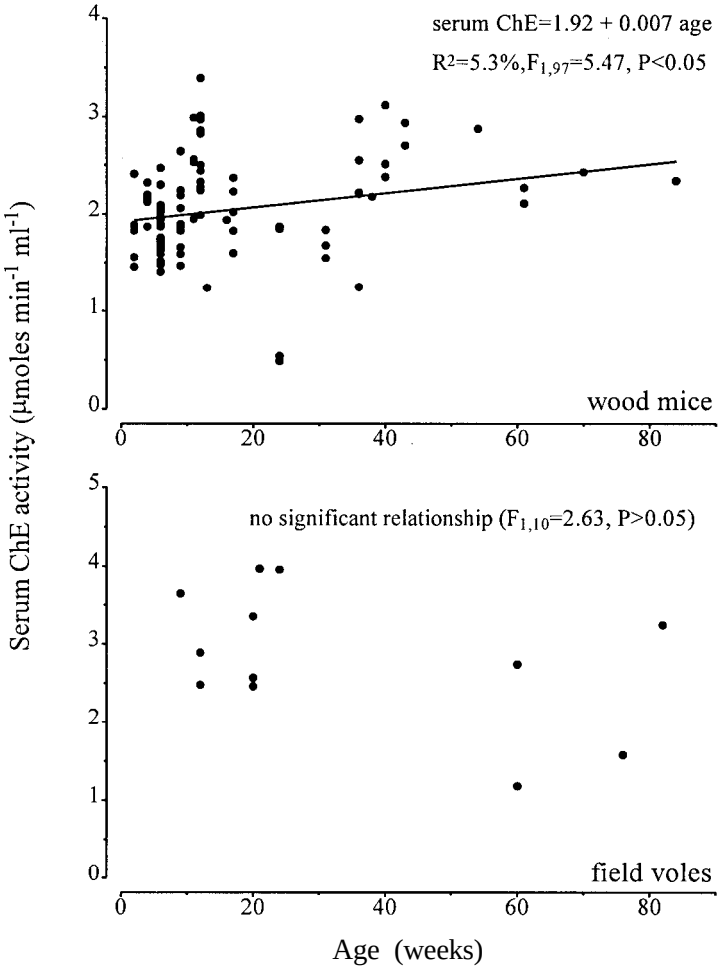

Figure 3. Variation in serum ChE activity with age in wood mice (sexes combined) and field voles.

trapped, juvenile (< 7 weeks old) wood mice should not be interpreted incorrectly as inhibition because juveniles are easily distinguished from adults by their pelage and low body weight.

Wood mice more than 7-10 weeks old all have adult pelage (Corbet and Harris 1991) and body weights (Shore *et al.* 1992) and so cannot easily be aged. Brain AChE activity does vary with age in animals over 7-10 weeks old (Figure 1) and this makes it difficult to define normal activity levels for adult mice. However, by combining data for males and females from the present study, the lower 99% confidence limit for normal brain AChE activity expected in an individual, adult (>10 weeks old) wood mouse could be calculated, irrespective of the sex or actual age of that animal. This lower

limit was 8.0 µmoles $min^{-1} g^{-1}$ and, assuming that ChE activities in captive-bred wood mice are typical of those in free-living individuals as comparison of the data from the present study with that from studies elsewhere suggests, this figure can be considered to be the lowest expected normal activity in free-living, adult wood mice. Thus, detection of brain AChE activities below 8.0 µmoles $min^{-1} g^{-1}$ in adult wood mice may indicate inhibition of activity by anticholinesterase pesticides or other factors. However, this value can only be used as a guide if the assay conditions employed are identical to those in the present study; optimal assay conditions, such as the substrate concentration needed to achieve Vmax, were not defined and variation in assay conditions may alter the values for AChE activity which are measured.

For field voles, the lower 99% confidence limit for expected brain AChE activity in an individual vole was 4.5 µmoles $min^{-1} g^{-1}$. This confidence limit was relatively wide compared with that for wood mice, reflecting that there was a more pronounced variation in activity throughout the adult part of the lifespan in voles. Thus, unless actual age is estimated in free-living voles, for instance by eye lens weight (Thomas and Bellis 1980), inhibition of brain AChE may only be inferred if activity, measured using the same assay conditions as in the present study, is lower than 4.5 µmoles $min^{-1} g^{-1}$.

The relative lack of variation in serum ChE activity with age or sex means that, in contrast to brain esterase activity, it may be possible to define normal serum activity values with tight confidence limits for animals of unknown age. This might allow more sensitive assessment of exposure to pesticides than is possible using measures of brain AChE activity. Preliminary studies have also indicated that measures of serum ChE inhibition might be used to predict the level of AChE inhibition in the brain following exposure to OP pesticides (Fishwick and Shore, *unpub. data*). Thus, it may be possible to use serum ChE activity both as a biomarker of exposure and a non-destructive predictor of the degree of inhibition in the brain. However, only acetylthiocholine iodide was used as a substrate in the present study and it is not known to what extent non-specific ChEs may have contributed to the ChE activity measured in the serum or how they may affect intra-species variation in normal activity levels. Further investigation both of normal serum activity using other substrates and of the relationship between serum and brain ChE activity in wild rodents is merited.

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