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Effects of acetylcholinesterase and butyrylcholinesterase inhibition on breathing in mice adapted or not to reduced acetylcholinesterase

Eliane Boudinot[a], Laurent Taysse[b], Sébastien Daulon[b], Arnaud Chatonnet[c], Jean Champagnat[a], Arthur S. Foutz[a,*]

[a]*Neurobiologie Génétique et Intégrative, Institut de Neurobiologie Alfred Fessard, C.N.R.S., 91198 Gif-sur-Yvette, France*
[b]*Centre d'Etudes du Bouchet, Vert-le-Petit, France*
[c]*Département de Physiologie Animale, I.N.R.A, Montpellier, France*

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Abstract

We investigated the contributions of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition to the respiratory dysfunction produced by organophosphates in mice which were adapted or not to low AChE activity. Effects of acute selective inhibition of AChE and BChE on ventilation measured by whole-body plethysmography were compared in mice with either normal AChE activity (wild-type), or mice adapted to a null AChE activity (homozygotes for AChE gene deletion) or adapted to an intermediate level of activity (heterozygotes). In wild-type mice acute reduction of AChE by Huperzine A (1 mg/kg) to the level found in asymptomatic heterozygotes, induced tremors but no respiratory depression, whereas the same dose of Huperzine in heterozygote animals further reduced AChE activity, increased tidal volume (V_T) and decreased breathing frequency (f_R). A lethal dose of Huperzine in wild-type mice augmented these respiratory effects, but was ineffective in homozygotes. BChE inhibition by bambuterol was ineffective in wild-type mice and heterozygotes, decreased V_T in homozygotes adapted to null AChE activity but increased V_T in wild-type mice acutely treated with Huperzine, also aggravating the cholinergic syndrome. We conclude that: (1) Huperzine does not perturb respiration at a dose inhibiting 40% of AChE, and at a lethal dose does not affect any other enzyme important for respiration; (2) Respiratory function is more sensitive to anticholinesterases in heterozygotes than in wild-type mice; (3) BChE may play distinct roles in respiratory function, because its inhibition has opposite effects on tidal volume depending on whether the mouse has adapted to null AChE or whether AChE has been lowered acutely; (4) BChE inhibition may contribute to the respiratory toxicity of organophosphates.

Keywords: Huperzine A; Bambuterol; Acetylcholinesterase; Butyrylcholinesterase; Respiration; Knockout; Mice

1. Introduction

A wide body of literature indicates that the respiratory system is a major target of organophosphate (OP) nerve agents, which inhibit the enzyme acetylcholinesterase (AChE). At toxic doses these agents produce a cholinergic syndrome (tremors, salivation, chewing, pinpoint pupil) and, at lethal doses, convulsions and respiratory failure (De Candole et al., 1953, Chang et al., 1990). Cholinergic mechanisms are involved in many aspects of respiratory function (reviews in Haji et al., 2000, Burton and Kazemi, 2000), all of which can be affected by OP-induced acetylcholine accumulation. Different OPs have indeed different toxicological profiles on respiration, depending in part on the predominance of central or peripheral effects (De Candole et al., 1953). However, while non-selective inhibition of other enzymes, notably butyrylcholinesterase (BChE), was shown to play a role in their toxicity (Marrs and Maynard, 1994; Duysen et al., 2001), it is not known how this might affect the respiratory function.

A widely accepted pre-treatment strategy against intoxication by OP nerve agents consists in chronic admin-

* Corresponding author. Tel.: +33 1 6982 3416; fax: +33 1 6982 4178.
E-mail address: foutz@iaf.cnrs-gif.fr (A.S. Foutz).

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istration of a reversible inhibitor of AChE at a dosage which sequestrates about 30% of the pool of AChE and shields it from irreversible inhibition by the toxic agent (Keeler et al., 1991). However, it is not known whether the respiratory response to acute AChE inhibition is affected when it occurs on a background of permanently low AChE activity.

An alkaloid isolated from the Chinese club moss *Huperzia serrata*, Huperzine A, has been shown to be a promising compound in the preventive management of OP intoxication (Lallement et al., 2001a,b, 2002a,b). Used as a pre-treatment to sequester a fraction of the AChE pool, Huperzine significantly decreases OP-induced mortality (Grunwald et al., 1994; Lallement et al., 2001b; Tonduli et al., 2001). However, its effects on respiratory function at therapeutic and toxic doses have not been evaluated.

A model for permanently reduced or null AChE levels has recently become available (Xie et al., 2000). Mice heterozygous for the AChE gene deletion present about one half of the normal amount of AChE, which allows one to investigate the effects of anticholinesterases on respiration when AChE activity is permanently reduced, while nullizygous mice completely lack AChE and can be used to reveal non-AChE enzymatic targets of OP compounds (Duysen et al., 2001). We used this model to investigate the following points: (1) Do the effects of toxic doses of Huperzine A on respiration, which we evaluated in normal wild-type mice, result from a selective inhibition of AChE? (2) Is the respiratory response to acute AChE inhibition affected when it occurs on a background of permanently low AChE activity? To this end, we first determined a dose of Huperzine which acutely reduced AChE activity in wild-type animals (presenting a normal level of AChE) to levels comparable to those found in mice heterozygous for the AChE gene deletion (presenting a permanently reduced level of AChE). We then compared the effects on respiration of this dose of Huperzine A in wild type and heterozygous animals. (3) Does BChE inhibition contribute to respiratory toxicity? Apart from its beneficial role as a scavenger for OPs, BChE has been shown to be essential for the survival of AChE knockout mice (Xie et al., 2000), because inhibition of this enzyme induces respiratory failure (Chatonnet et al., 2003). To distinguish whether BChE plays a substitutive role to AChE as has been suggested (Mesulam et al., 2002), or a different role, in respiratory mechanisms in vivo when AChE is inactivated, we used two approaches. We first determined whether the effects of selective AChE inhibition by Huperzine in wild-type mice were similar or not to those produced by a selective BChE inhibitor, bambuterol, in AChE knockout mice. Then, to distinguish whether acute AChE and BChE inhibition could have cumulative effects which would indicate similar mechanisms of action, in wild-type mice we compared the effects of AChE inhibition, BChE inhibition, and a combination of both.

2. Materials and methods

2.1. Animals

Animal studies were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), and French Government animal protection laws. Potentially lethal doses of drugs were given to the smallest possible number of adult animals. An AChE knockout mouse colony (Xie et al., 2000) was maintained under standard conditions in a transgenic mouse facility by breeding heterozygous males and females. Original founders were provided by Prof. O. Lockridge (Eppley Institute, Omaha, Nebraska) and were maintained in a 129SVJ strain background. Feeding the nullizygous mice with an enriched liquid diet allows some animals to survive past the weaning period into adulthood (Duysen et al., 2002). Mice of either sex were used between 1 and 5 months of age, and were genotyped as described previously (Chatonnet et al., 2003). The experiments comparing separate and combined AChE and BChE inhibition in wild-type mice were carried out in standard Swiss mice.

2.2. In vivo measurement of ventilation

Breathing activity was measured using a barometric method (Bartlett and Tenney, 1970). The plethysmograph chamber (700 ml), equipped with a temperature sensor, was connected through a slow leak to a reference chamber, and the pressure difference between the two chambers was measured with a differential pressure transducer (Validyne, DP-103-10) connected to a sine wave carrier demodulator (Validyne, CD15). The spirogram was stored on a PC computer (CED interface). Calibrations were made during each recording by injecting 100 μl of air into the experimental chamber with a syringe.

In the recording chamber the mice were partially restrained by the tail with a thin temperature probe permanently inserted in the rectum as described previously (Boudinot et al., 2004b). The chamber was maintained at 27–28 °C and was permanently flushed with fresh humidified air between the 2–3 min data collection sessions during which time it was hermetically sealed.

In each sample, periods of baseline breathing were analyzed as described previously (Boudinot et al., 2004a). A computer program (ACQUIS1 software, BioLogic, Claix, France) measured the duration of inspiration (T_I) and expiration (T_E), ventilatory frequency (f_R), tidal volume (V_T) and minute ventilation (V_E). Mean values were calculated from breaths collected within a minimum period of 15 s.

Drugs or the vehicle (saline) were injected subcutaneously (1 ml/100 g) after baseline breathing had been measured.

2.3. Enzyme activity assays

For cholinesterase assays, mice were killed by cervical dislocation, without prior treatment or at the end of the recording session following drug injection. Whole brains were frozen in isopentane and stored at −80 °C. Nervous tissue was weighed and homogenized in 20 volumes of ice-cooled 0.1 M sodium phosphate buffer (pH 8) with 1 M NaCl, 1% Triton X-100, using a homogenizer (Ultra turrax-Ika Werk). AChE activity was measured by the method of Ellman et al. (1961) at 37 °C in a temperature-controlled spectrophotometer (Beckman Instruments). Samples were pre-incubated with 5,5-dithio-bis(2-nitrobenzoic acid) in 0.1 M phosphate buffer, pH7, to react free sulfhydryl groups before addition of substrate. Total cholinesterase activity was assayed with 1 mM acetylthiocholine and was considered to be AChE activity because butyrylcholinesterase activity was comparatively negligible (Chatonnet et al., 2003). The enzymatic activity was expressed in international units (IU)/L.

2.4. Drugs and data analysis

(−)Huperzine A was purchased from Sigma-Aldrich. Bambuterol was a generous gift from Astra-Zeneca France.

The results, expressed as means±SEM, were analyzed using one-way or two-way repeated-measures analysis of variance (ANOVA) followed by Bonferroni-Dunn post hoc tests, or by unpaired *t*-tests.

3. Results

3.1. Acute inhibition of AChE in AChE+/+ and +/− mice

Whole brain AChE activity was much lower in heterozygous AChE+/− mice than in wild-type AChE+/+ mice, and was null in homozygous AChE−/− animals (Fig. 1). These findings are consistent with previous studies (Xie et al., 2000) . Huperzine A (1 mg/kg) decreased AChE activity in both AChE+/+ mice (by 42%) and in AChE+/− mice (by 54%). After Huperzine treatment, AChE activity in AChE+/+ animals was not significantly different from activity in untreated AChE+/− animals (Fig. 1). However, despite a comparable level of AChE activity, Huperzine-treated AChE+/+ animals showed a cholinergic syndrome with tremors whereas untreated AChE+/− mice were asymptomatic, thus indicating adaptation to low AChE in these animals. AChE+/− animals treated with Huperzine (1 mg/kg) presented a cholinergic syndrome but no mortality, although AChE residual activity was 29% of activity in untreated AChE+/+ mice (Fig. 1). However, when AChE was abruptly decreased in AChE+/+ mice (not from a background of reduced activity), a larger residual activity, 42%, did not prevent lethality after a 6 mg/kg dose of Huperzine.

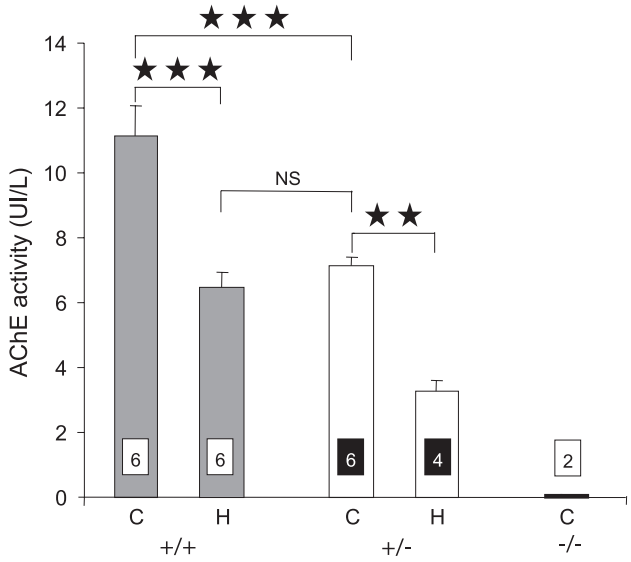

Fig. 1. Acetylcholinesterase (AChE) activity and effects of Huperzine A in wild-type (+/+), heterozygous (+/−) and homozygous (−/−) mice for AChE gene deletion. C, untreated; H, Huperzine A (1 mg/kg). The number of animals is indicated in the bar graphs. $^{**}P<0.01$; $^{***}P<0.001$.

3.2. Low respiratory toxicity of Huperzine A in wild-type mice

In wild-type AChE+/+ animals a dose of Huperzine A (1 mg/kg) which elicited a cholinergic syndrome with tremors and salivation (within 5 min), and prostration (within 10 min), did not provoke any significant change in minute ventilation (V_E), breathing frequency (f_R) and tidal volume (V_T) within one hour, compared with the same animals injected with the vehicle on a previous day (Fig. 2, left traces). In two animals larger doses of Huperzine (2–3 mg/kg) transiently decreased breathing frequency (by 36–50%) and increased tidal volume (by 62–137%) within 30 min after injection, but within 90 min respiration had returned to normal (not shown).

3.3. Selectivity of Huperzine A: lethal doses in wild-type mice are ineffective in AChE−/− mice

After observing that the 2 mg/kg dose was ineffective in a AChE−/− mouse, we administered Huperzine to AChE+/+ mice (n=3) at a dose of 6 mg/kg (2 LD_{50} for sc injection, see Zangara, 2003) which was lethal within 20 min. The final disorganization of respiratory activity in the last minute before death was preceded by a sharp increase in tidal volume (from 9.97±0.29 to 35.7±9.9 μl/g) and a decrease in breathing frequency (from 161±22 to 55±11 breaths/min). This dose of Huperzine did not produce any behavioral or respiratory effects in AChE−/− mice (Fig. 3) thus indicating that the effects of Huperzine on respiration result from a selective targeting of AChE.

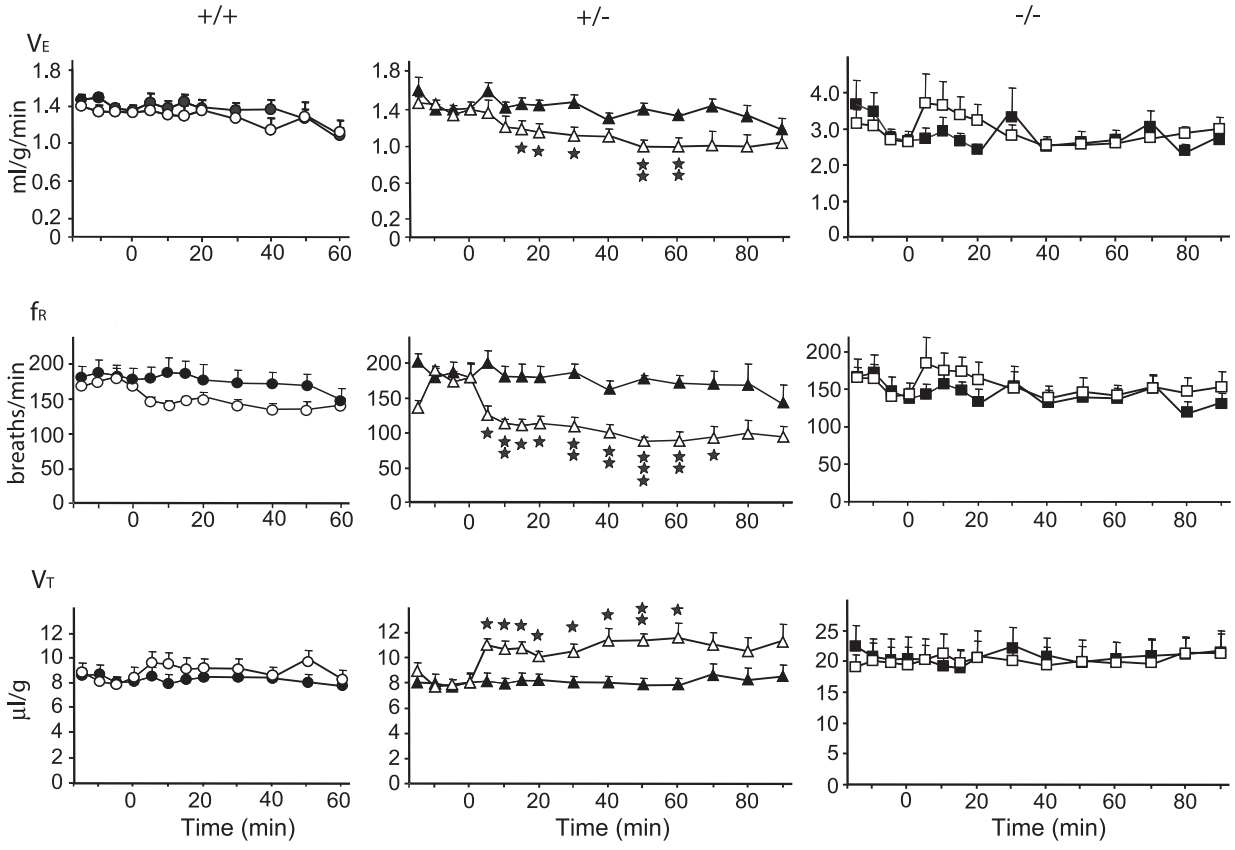

Fig. 2. Effects of Huperzine A (1 mg/kg) on minute ventilation (V_E), breathing frequency (f_R) and tidal volume (V_T) in AChE+/+ mice (left graphs, n=6), AChE+/− mice (middle graphs, n=4) and AChE−/− mice (right graphs, n=6). Note the reduction in f_R and the increase in V_T in AChE+/− mice, and the ineffectiveness of Huperzine in both AChE+/+ and AChE−/− animals. The same animals were injected with the vehicle (black symbols) or Huperzine (open symbols) at time 0. *P<0.05; **P<0.01; ***P<0.001.

3.4. Effects of acute inhibition of AChE on respiratory activity and core temperature: dependence on the pre-existing level of AChE

In AChE+/− animals, which are behaviorally normal, the 1 mg/kg dose of Huperzine produced cholinergic symptoms as in the wild-type mice, but it also affected respiration. Breathing frequency decreased and tidal volume increased, which resulted in a slight decrease in ventilation (Fig. 2 middle traces). These ventilatory effects are qualitatively similar to those produced by larger doses of Huperzine (2–3 mg/kg) in wild-type animals, and indicate that mice are more sensitive to a given does of anticholinesterase when they have a low pre-existing level of AChE.

In AChE−/− animals presenting a null AChE activity, the same dose of Huperzine (1 mg/kg) did not induce any visible syndrome nor did it affect respiration (Fig. 2, right traces).

After injection of Huperzine (1 mg/kg) , rectal temperature steadily decreased in AChE+/+ and AChE+/− mice compared to the same animals injected with saline, but did not change in AChE−/− animals. Sixty minutes after injection of Huperzine or saline, core temperatures were respectively 35.1±0.4 °C versus 36.7±0.3 °C in AChE+/+ mice and 34.3±0.6 °C versus 37.5±0.3 in AChE+/− mice (for both genotypes P<0.01, paired t-tests), whereas in AChE−/− animals rectal temperatures were respectively 36.6±0.2 °C versus 36.4±0.3 °C, an insignificant change.

3.5. Can BChE substitute for AChE in AChE−/− mice?

The availability of AChE−/− mice and the demonstration that Huperzine affects respiration through selective inhibition of AChE in wild-type mice, allowed us to test whether or not BChE substituted for the catalytic function of AChE in AChE−/− mice. If this hypothesis is correct, one would expect an acute inhibition of AChE in wild-type mice and an acute inhibition of BChE in AChE−/− mice would produce the same effects on respiratory muscle contraction and consequently on tidal volume.

A first step was to verify that large doses of the selective BChE inhibitor bambuterol did not affect ventilation in mice with functional AChE. We have reported that bambuterol does not affect breathing in heterozygote animals (Chatonnet et al., 2003). In wild-type mice (n=3), a large dose of bambuterol was ineffective. This dose (2 mg/kg s.c.) was much higher than the dose (0.05 mg/kg) previously shown to inhibit 94% of plasma BChE in mice (Chatonnet et al., 2003). At 30 min, tidal volume (µl/g) was 8.27±0.23 vs.

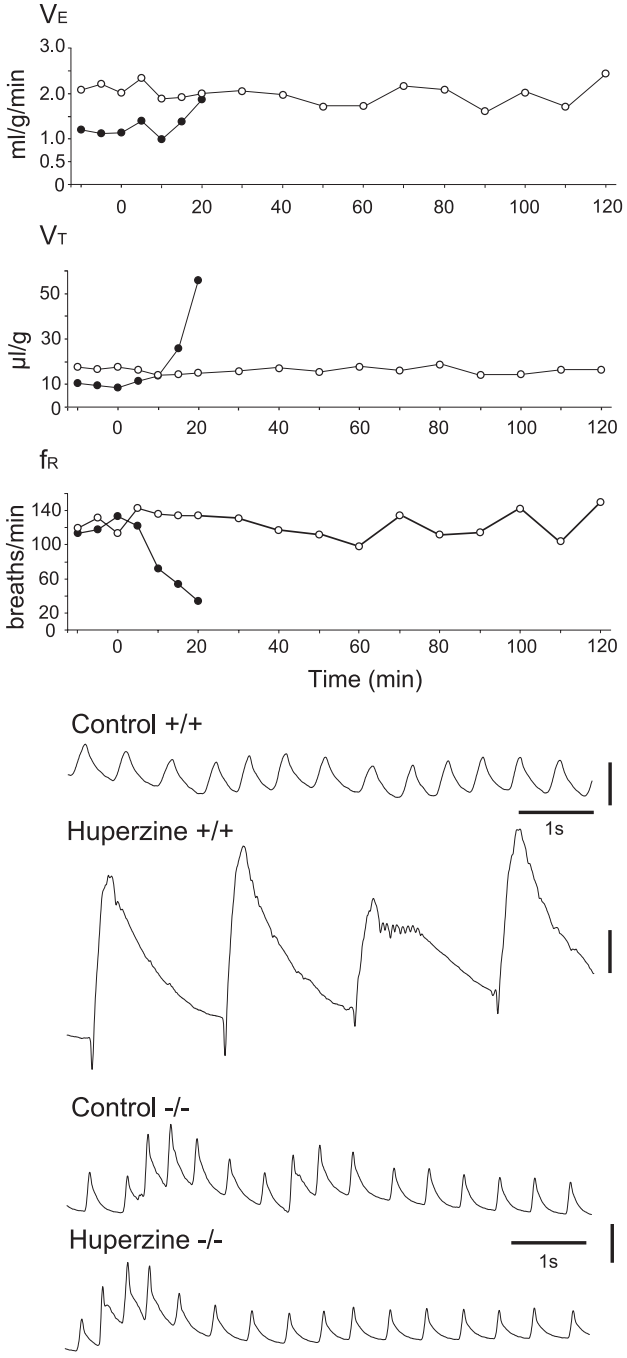

Fig. 3. Effects of a lethal dose of Huperzine (6 mg/kg) in a AChE+/+ mouse and a AChE−/− mouse. Upper graph: In the AChE+/+ mouse (black symbols) f_R decreased and V_T increased rapidly, whereas respiration of the AChE −/− mouse (open symbols) was unaffected. Lower traces: respiration records before Huperzine injection the wild-type mouse (control +/+) and AChE−/− mouse (control −/−), and 20 min after injection in the wild-type mouse (Huperzine +/+) and 30 min after injection in the AChE−/− mouse (Huperzine −/−). Note the ineffectiveness of Huperzine in the AChE−/− mouse. Vertical calibration bars: 200 µL, inspiration upward deflection.

8.44±0.42 before injection, breathing frequency (breaths/min) was 203±17 vs. 210±13, and ventilation (ml/g/min) was 1.67±0.13 vs. 1.76±0.21. Thus BChE inhibition does not affect breathing when AChE is partly or completely functional.

3.5.1. Opposing effects of AChE inhibition on tidal volume in wild-type mice and of BChE inhibition in AChE−/− mice

At effective doses for the respective genotypes, Huperzine increased tidal volume in AChE+/+ mice (Fig. 3), as well as in AChE+/− mice (Fig. 2, middle traces). In AChE−/− mice however, a small dose of bambuterol (25 µg/kg) decreased

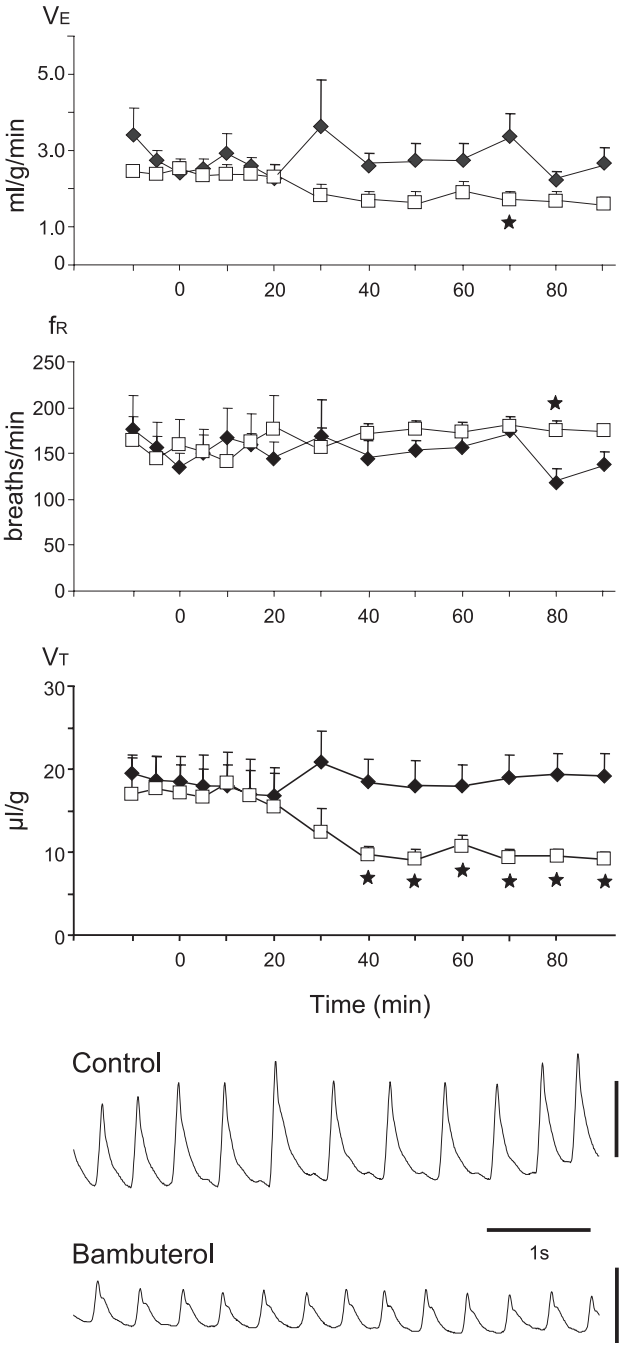

Fig. 4. Effect of a sub-lethal dose of bambuterol (25 µg/kg) on respiration in AChE−/− adult mice. Black symbols: control injection of vehicle at time 0 (n=4). Open symbols: same animals with bambuterol administration. Lower traces: plethysmographic records of respiration 30 min after injection of vehicle (control) or bambuterol. Note the profound decrease in tidal volume with no change in frequency. Vertical calibration bars: 200 µL, inspiration upward deflection.

tidal volume with no change in breathing frequency (Fig. 4). These results indicate that AChE inactivation and the consequent ACh accumulation in wild-type animals increases muscle contraction (essentially the diaphragm) and augments the inspiratory effort (tidal volume), but that BChE inactivation in animals adapted to a null AChE activity reduces muscle contraction, an effect incompatible with a (predominantly) substitutive role for AChE function.

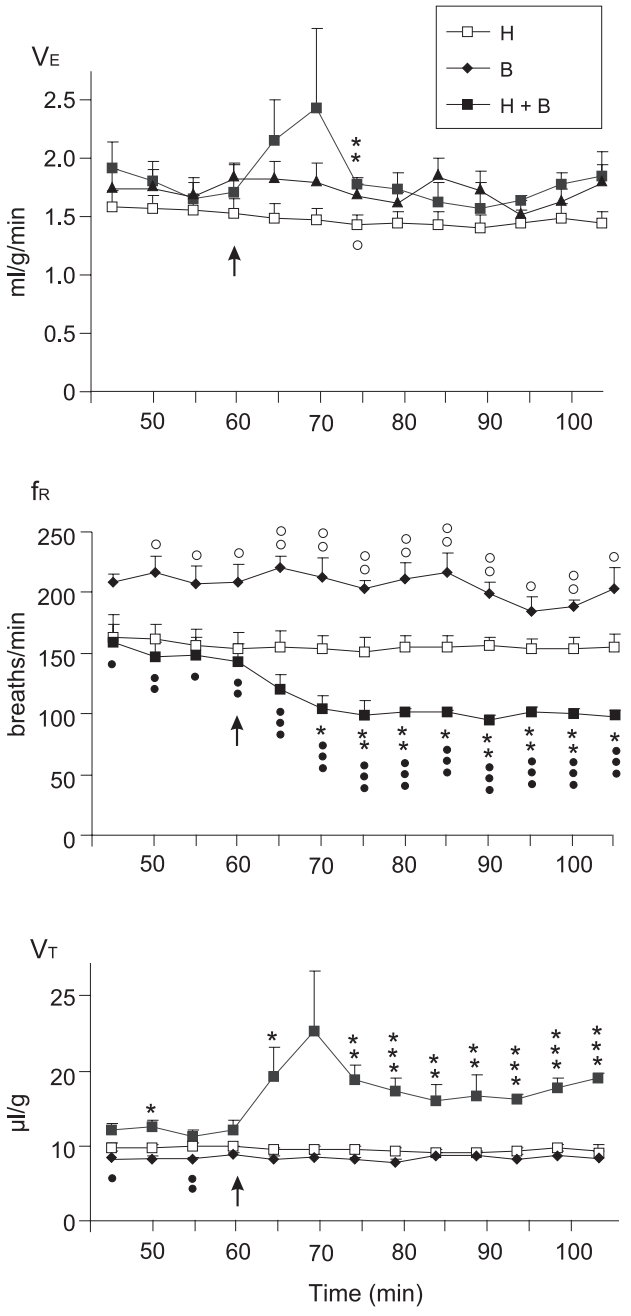

Fig. 5. Acute inhibition of AChE, BChE or both on respiration in wild type mice. Effects of Huperzine A alone (1 mg/kg, n=4), Bambuterol alone (0.2 mg/kg, n=3) and Huperzine followed by Bambuterol (n=6) on minute ventilation (V_E), breathing frequency (f_R) and tidal volume (V_T). Note the decrease of f_R and the increase of V_T when Bambuterol is injected after Huperzine. Huperzine (H) was injected at time 0, Bambuterol (B) at 60 min (arrow). *°P<0.05; **°°P<0.01; ***°°°P<0.001. Asterisks: B+H versus H; open circles: B versus H; filled circles: H+B versus B.

3.6. Acute BChE inhibition enhances the cholinergic effects of acute AChE inhibition in wild-type mice

We investigated whether BChE inhibition potentiates the effects of AChE inhibition, which would suggest that BChE substitutes for the catalytic role of AChE when this enzyme is acutely inhibited. In three groups of wild-type mice we administered Huperzine alone (1 mg/kg), Bambuterol alone (0.2 mg/kg, one-tenth of the dose shown to be ineffective in wild-type mice), or Bambuterol 60 min after Huperzine (Fig. 5). Although both drugs administered separately did not affect respiration, the small dose of bambuterol given after Huperzine produced an intense salivation, convulsions, and the respiratory symptoms of a Huperzine overdose (decreased frequency, increased tidal volume) within 10 min. One half of the animals (3/6) died within 25 min (the others were sacrificed at the end of the recording). Thus acute AChE and BChE inhibition act synergistically, which is suggestive of a common functional role of the two enzymes.

4. Discussion

The results show that Huperzine has a low respiratory toxicity and specifically targets AChE. This selectivity allowed us to show that the less AChE an animal carries, the more sensitive is its respiratory function to the same dose of anticholinesterase. In the absence of AChE, respiratory toxicity results from inhibition of BChE. BChE inhibition has opposite effects on tidal volume depending on whether the mouse has adapted to null AChE or whether AChE has been lowered acutely, which is suggestive that two distinct functions are exerted by BChE.

4.1. Technical considerations

The pressure signal in the closed plethysmograph is not only related to tidal volume, but also to changes in flow resistance (Mortola and Frappell, 1998; Enhorning et al., 1998). Since AChE inhibitors are known to produce bronchoconstriction (De Candole et al., 1953), the drug-induced increase in tidal volume observed in this study might have been overestimated. However our results are consistent with the large increase in the inspiratory discharge recorded from the phrenic nerve (Foutz et al., 1989) and from the diaphragm (Chang et al., 1990) at the onset of OP poisoning.

4.2. Low respiratory toxicity of Huperzine A in wild-type animals

Huperzine A is a slow, reversible inhibitor of AChE, with a Ki for butyrylcholinesterase (BChE) three orders of

magnitude higher (Ashani et al., 1992), an interesting property which preserves the scavenging capacity of BChE for the toxic agent (Raveh et al., 1993). Furthermore, Huperzine A has little direct effect on cholinergic receptors as compared to other cholinesterase inhibitors (Tang et al., 1989), but its antagonistic effects on *N*-methyl-D-aspartate (NMDA) receptors might contribute to its neuroprotective properties (Zangara, 2003). Huperzine and its derivatives could also be of interest in the treatment of neurological disorders such as Alzheimer's disease (Camps and Munoz-Torrero, 2001).The present results show that a dose of Huperzine A (1 mg/kg), reducing AChE activity by at most one half, provoked a cholinergic syndrome and hypothermia, a well-described effect of anticholinesterases in rodents (Gordon, 1996), but did not alter ventilation in wild-type animals. Given that doses of Huperzine used for neuroprotection against chemical warfare agents do not exceed 0.5 mg/kg (Grunwald et al., 1994; Tonduli et al., 2001; Lallement et al., 2002a,b), our results suggest that Huperzine has a satisfactory safety margin with respect to respiratory function.

4.3. Respiratory consequences of acute versus permanent decrease in acetylcholinesterase activity

In wild-type animals Huperzine-induced reductions in AChE activity to levels found in heterozygotes did not affect respiration but consistently provoked a cholinergic syndrome which was never observed in untreated heterozygotes. These heterozygotes, however, despite their permanently low AChE activity, show neither a behavioural phenotype (Duysen et al., 2002) nor a modification of breathing parameters (Emery et al., 2001; Boudinot et al., 2004a,b). This indicates that heterozygotes have completely adapted functionally to permanently low levels of AChE, which when induced acutely by pharmacological means in wild-type animals, produce a cholinergic syndrome. Administered to heterozygotes, however, the same dose of Huperzine which had no respiratory effect in wild-type mice affected respiration. This indicates that the large "safety margin" which allows wild-type animals to tolerate around a 30% decrease in AChE without consequences does not exist in heterozygotes adapted to low AChE levels. Hence the less AChE a mouse carries, the more sensitive it is to anticholinesterase. These results show that reducing levels of AChE alone can explain, at least partially, the greater sensitivity of heterozygotes to the toxic effects of OP compounds (Xie et al., 2000; Duysen et al., 2001). Non-specific effects of these compounds on butyrylcholinesterase might not be involved in the enhanced toxicity in heterozygotes, because bambuterol, which profoundly inhibits butyrylcholinesterase in mice (Chatonnet et al., 2003), has no respiratory toxicity in heterozygous animals. Since it kills homozygotes, it is likely that BChE inhibition becomes critical only in animals which have no AChE at all.

The survival of all heterozygotes treated with Huperzine and reaching levels of AChE activity which induce mortality in wild-type animals can be explained by their prior adaptation to permanent excesses of ACh through down-regulation of ACh receptors. We have shown that in AChE−/− mice respiratory neurons and motoneurons adapt to the complete absence of AChE by down-regulating their response to cholinergic agonists (Chatonnet et al., 2003). Down-regulation of muscarinic receptors also occurs in other brain structures in these mice (Bernard et al., 2003; Volpicelli-Daley et al., 2003) and has been also shown in heterozygous mice (Li et al., 2003). There is also good evidence that adaptation to excess ACh needs not occur during embryonic or fetal life. In adult wild-type animals internalisation of muscarinic receptors occurs after acute exposure to organophosphorus anticholinesterases (Liste et al., 2002) and receptor desensitization occurs within minutes upon bath application of acetylcholine agonists in the isolated mouse brainstem (Chatonnet et al., 2003). The present results show that combining acute AChE inhibition and a background of reduced AChE activity, allows survival with a modest alteration of respiration, despite residual AChE activity (29% of the activity in untreated wild-type) lower than the threshold (35%) below which seizures occur following acute AChE inactivation (Tonduli et al., 1999). This is also in keeping with earlier assertions that, providing that some recovery time is allowed under respiratory assistance (and/or anesthetics not administered), respiration is compatible with very low levels of AChE activity (Meeter and Wolthuis, 1968; Foutz et al., 1987, 1989).

In practical terms, the greater sensitivity of the respiratory function to a given dose of anticholinesterase in heterozygotes compared to wild-type (despite the tolerance of these animals to a low AChE level), suggests that the protective effects of chronic AChE inactivation might no longer be beneficial when chronic inactivation exceeds 40% (alternatively, the AChE threshold for breathing dysfunction might not correlate with the lethal threshold). This point deserves further investigation.

4.4. Acetylcholinesterase versus butyrylcholinesterase (BChE) inhibition

Huperzine A is a very selective inhibitor of AChE which penetrates the blood–brain barrier, has a selectivity ratio of 10^3 over BChE (Ashani et al., 1992), and does not affect BChE at doses that provide strong neuroprotection (Lallement et al., 2001b). We have shown here that Huperzine at toxic doses in heterozygous and wild-type animals increased tidal volume and decreased breathing frequency. This effect resulted from a selective inhibition of AChE because a dose that killed wild-type animals had no effect in AChE−/− mice. These mice showed no change in breathing pattern, no enhancement of body tremors, and no core hypothermia. Thus hypothermia, a common effect of anticholinesterases in rodents (Gordon, 1996), also resulted from a selective

inhibition of AChE but not other enzymes. This innocuousness of Huperzine in AChE−/− mice contrasts with the lethal effects of the non-selective OP compounds DFP and VX in these animals (Xie et al., 2000; Duysen et al., 2001), which can be attributed to inhibition of BChE because a selective inhibition of this enzyme by bambuterol resulted in a lethal respiratory arrest in AChE−/− mice (Chatonnet et al., 2003) but not in AChE+/+ and +/− animals. The effects of Huperzine were thus compared to those of bambuterol, a terbutaline carbamate prodrug, which is a very potent inhibitor for BChE with a selectivity ratio of about 10^4 over AChE (Tunek and Svensson, 1988). Bambuterol poorly penetrates the blood–brain barrier (Svensson, 1991) and we verified that doses of up to 0.5 mg/kg profoundly decrease plasma but not brain BChE activity (Chatonnet et al., 2003). This indicates that the effects of bambuterol observed in nullizygous mice arise from peripheral effects at the neuromuscular junction. Partial inhibition of BChE produces very different respiratory modifications than that produced by AChE inhibition in wild-type and heterozygous animals. The opposing effects of Huperzine and bambuterol on tidal volume show that if BChE has indeed a function in AChE-null mice, it is different from that of AChE at the post-synaptic membrane. Taken together, the present results demonstrate in vivo the selectivity of Huperzine for AChE. A recent study indicates that it may regulate ACh release presynaptically (Minic et al., 2003), which could explain the decrease in inspiratory effort (tidal volume) when it is inactivated in homozygous animals.

When AChE is inhibited acutely in wild-type mice, BChE inhibition aggravates the cholinergic effects. Given the low affinity of Huperzine for BChE, It is unlikely that this effect resulted from the suppression of a scavenger function of BChE for Huperzine, allowing more of the drug to inhibit AChE. A common catalytic function for the two enzymes at the post synaptic level is more likely, as suggested by a study of diaphragmatic contractility in AChE knockout mice (Adler et al., 2004). Taken together, the present results suggest two functionally opposite roles for BChE, each role being predominant under specific experimental conditions (acute decrease of AChE or chronic absence of AChE).

BChE is not the only possible secondary target of cholinesterase inhibitors. Compounds such as eserine and tacrine were recently shown to have non-AChE targets in a model of AChE-deficient zebrafish embryo naturally devoid of BChE (Behra et al., 2003). The ineffectiveness of Huperzine A in AChE−/− mice which are sensitive to BChE inhibition, shows that it did not target other enzymes important for respiratory function.

4.5. Conclusions

We conclude from these studies that (1) Huperzine A does not alter respiration at doses which produce behavioural symptoms, and does not target any other enzyme crucial for normal respiration; (2) Animals with permanently low levels of AChE activity have adapted, and tolerate a profound acute decrease in AChE activity with mild respiratory effects; (3) BChE may play functionally opposite roles in neurotransmission to respiratory muscles, possibly involving control of ACh release and ACh degradation; (4) BChE inhibition may contribute to the acute toxicity of organophosphate compounds. Taken together, our results suggest that BChE is an important enzyme in respiratory function, and that it should be left unaffected by medications designed to preventively sequester a fraction of the AChE pool.

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