Reduced nicotinic receptor function in sympathetic ganglia is responsible for the hypothermia in the acetylcholinesterase knockout mouse

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Cholinergic signalling in the sympathetic ganglia (SG) contributes to non-shivering thermogenesis by relaying the activation signal from the brain to SG neurons which activate many peripheral tissues to produce heat. Paradoxically, acetylcholinesterase (AChE) inhibitors, which should enhance cholinergic signalling, induce hypothermia. To understand the mechanism of how cholinergic signalling in the SG controls thermoregulation, we analysed infant AChE knockout mice, which are known to show hypothermia by postnatal day 15. Nicotinic receptor currents were reduced in acutely dissociated SG neurons of the AChE-deficient mice by over 40% compared with wild-type mice. When wild-type neurons were treated for 1 h with either oxotremorine-M, a muscarinic agonist, or nicotine, the amplitude of nicotinic receptor currents was also decreased by 40%. The hypothermia in AChE mutant mice was fully rescued by a peripheral injection of both ivermectin, which increases nicotinic receptor currents, and methyl-atropine, a muscarinic antagonist. Our results demonstrate that the hypothermia induced by the lack of AChE activity is primarily caused by a downregulation of nicotinic receptors via prolonged stimulation of muscarinic and nicotinic receptors in SG neurons. The stationary noise analysis of the nicotinic receptor current traces showed that the properties of single-channel activities were not different between the two genotypes, suggesting that the primary reason for downregulation of nicotinic receptors is due to a reduction of the receptors on the surface.

(Resubmitted 31 August 2006; accepted after revision 9 October 2006; first published online 12 October 2006)
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Non-shivering thermogenesis by heat production in brown adipose tissue (BAT) serves to protect the animals from exposure to cold and to regulate energy balance in small animals and infants of large animals (Lowell & Spiegelman, 2000). Signals arising from an exposure to cold are processed by the hypothalamus, leading to an activation of the efferent pathway controlling energy dissipation. This thermoregulatory signal is mediated via cholinergic nerve fibres of the preganglionic neurons that terminate at the sympathetic ganglion (SG) neurons (Cannon & Nedergaard, 2004). The SG neurons relay the signal to BAT by releasing noradrenaline, which in turn activates primarily β_3-adrenergic receptors. Activation of β_3-adrenergic receptors eventually leads to heat generation by utilization of the uncoupling protein 1 (UCP1) in BAT (Lowell & Spiegelman, 2000). The activation of SG neurons also leads to the activation of many peripheral thermogenic effectors, including the cutaneous blood vessels, sweat glands, skin and muscles (Morrison, 2001; Martini, 2004).

It has been demonstrated that the thermogenesis by the activation of BAT plays a critical role in infants, in contrast to adults. In adults, the control of the body temperature is achieved mainly by the hypothalamus, which creates a balance between heat gain and heat loss (Boulant, 2000). In infants, the physiology of the response to cold stress is more dependent on the metabolism of BAT in the periphery due to the immaturity of those effectors (Blackburn, 2003), which makes infants more prone to hypothermia. Consequently, the cholinergic system in infants might have a stronger contribution to thermogenesis due to these anatomical differences in infants.

The SG neurons are at the centre of the cholinergic signalling pathway that leads to heat generation by BAT and other effectors. Various nicotinic receptor channels (Skok, 2002) and muscarinic receptors (Del Rio *et al.* 1999; Shapiro *et al.* 2001) are expressed in SG neurons to transmit the thermoregulatory signal. The released acetylcholine (ACh) is removed via hydrolysis by acetylcholinesterase

 DOI: 10.1113/jphysiol.2006.120147

(AChE) at the synaptic cleft. The enhancement of cholinergic signalling in SG neurons by inhibition of AChE is expected to strengthen the heat generation by increased activation of nicotinic and muscarinic receptors. Paradoxically, however, agonists of nicotinic and muscarinic receptors, or AChE inhibitors, such as nerve gases and pesticides, have been shown to induce hypothermia (Clement, 1993; Gordon & Grantham, 1999). Furthermore, in a recent report, the AChE knockout mouse was shown to display hypothermia during the infant period (Duysen *et al.* 2002). Although the main target of these various agonists and antagonists seems to be at the cholinergic system, it is unknown how the molecular components of the cholinergic system, i.e. nicotinic receptor, muscarinic receptor and AChE, interact with or modulate each other to control thermoregulation at the level of SG.

To investigate the thermoregulatory function of the cholinergic system in the SG, we have generated and utilized infant AChE knockout mice that display hypothermia only during the infant period ranging from birth to postnatal day (PND) 15.

Methods

Generation of AChE knockout mice

Mouse DNA of the AChE gene sequence corresponding to 549–903 bp was amplified by genomic DNA PCR, and was used for isolating mouse genomic DNA clones containing the AChE locus from the phage library. The targeting vector containing 8.4 kb homologous fragments with double selection markers, neo and diphtheria toxin-A, was made as shown in Supplemental Fig. 1A and introduced into J1 embryonic stem (ES) cell lines derived from the 129/sv strain. The targeted ES clones were identified by Southern blot analysis and used for Cre transfection to delete the NEO-IRES-TK selection marker. Depending on the use of *loxP* recombination sites (Sauer, 1998), ES clones containing neither selection markers nor exon3 were obtained and used in the generation of germline chimeras, as previously described (Kim *et al.* 1997). Male germline chimeras were crossed with female C57BL/6J mice to obtain F1 heterozygotes (AChE+/–) and these F1 were intercrossed to obtain F2 homozygous mutant mice (AChE–/–). This knockout mouse line was maintained in two inbred strains, C57BL/6J and 129/sv. To make the genetically homogeneous F1 mice, we mated each heterozygous mouse in C57BL/6J and 129/sv that had a uniform genetic background through several backcrosses. We did not observe any difference in our results for survival rate and body weight measurement between F1 homozygotes and F2 homozygotes, and we collected the data from experiments using F1 and F2 mice. No sex bias was observed in the offspring, and the expected Mendelian ratio was observed among wild-type, and heterozygous and homozygous mutant mice. The genotypes were determined by PCR analysis using the primers: F3, 5′-CCGGAGGCTCTCATCAATAC-3′; B3, 5′-TCATTCTGTCGTGTCCAAGC-3′; W3, 5′-GAGGC-ACGGTGTTCAAAGAT-3′. The wild-type locus yielded an 800 bp fragment amplified by the F3 and W3 primers, whereas the mutant locus yielded a 400 bp fragment amplified by the F3 and B3 primers. Animal care and handling were carried out following institutional guidelines (KIST, Seoul, Korea). The mice were maintained with freely accessible food and water under a 12:12 h light:dark cycle, with the light cycle beginning at 06.00 h.

AChE enzyme activity assay

AChE activities in the brain regions were determined according to the method of Ellman *et al.* (1961). Whole brain was homogenized at a concentration of 40 mg ml^{-1} in 50 mM Tris-HCl (pH 7.7) in a glass mortar using a Wheaton motorized tissue grinder and a Teflon pestle, and centrifuged at 5000 *g* for 5 min (4°C) to obtain a crude membrane pellet. The supernatant was discarded, and the pellet was resuspended in the original volume of the same buffer. This preparation was used to eliminate potential contributions from plasma butyrylcholinesterase. All samples were stored at −70°C until use. All tissue supernatants were diluted 1:10 with 50 mM Tris-HCl buffer. Diluted supernatant (1 ml) was used for AChE activity assay in a total volume of 2 ml of 50 mM Tris-HCl buffer containing 0.3 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and 0.02 mM ethopropazine. After incubation for 10 min at 37°C, the reaction was started by the addition of 0.6 mM acetylthiocholine iodide. A blank contained buffer in the place of substrate, and the enzyme activity was monitored by recording the absorbance at 412 nm (Richardson *et al.* 2001). AChE enzyme activity was calculated as change in absorbance (ΔA) per minute. Protein concentration was determined by the Bradford method.

Body weight measurement

AChE mutant mice and wild-type littermates were subjected to body weight measurements every day. After the weaning day (PND19), the mutant mice were transferred to an Environment Control Clean Rack (Three shine, Korea), which maintains a constant ambient temperature and humidity. The AChE mutant mice were kept at 29 ± 1°C ambient temperature and 50 ± 5% humidity to PND35. Both mutant and wild-type mice were fed with solid pellet food.

Body temperature measurement

The surface body temperature of mice was measured with a digital thermometer, HANNA model HI8757, and surface microprobe TP-K03, Type K thermocouple (TES, Taiwan). A surface probe was used because very young mice were too small to allow the use of an anal probe (Duysen *et al.* 2002). Starting from 5 min after separating mice pups from their mother and isolating them at room temperature ($23 \pm 1^{\circ}$C), the body temperature was measured periodically for 6.5 h. All drugs were injected intraperitoneally and administered 15 min after separation from the parental cage.

Whole-cell patch clamping

All experiments were carried out using cells acutely isolated from PND10 mice. Mice were killed with an overdose of Avertin applied intraperitoneally. SG from L3–L5 regions were dissociated using the method previously described, with some modifications (Namkung *et al.* 1998; Raman & Bean, 1999; Morrison *et al.* 2000). Briefly, the SG neurons were excised and incubated at 37°C in Hanks' buffered solution containing 1.5 mg ml^{-1} collagenase and 6 mg ml^{-1} albumin for 15 min, followed by an incubation in 1 mg ml^{-1} trypsin for 30 min. Cells were triturated with fire-polished Pasteur pipettes and then plated onto poly-D-lysine-coated coverslips. Nicotinic receptor currents were recorded under voltage-clamp configuration. The patch electrodes were filled with a solution containing (mM): 125 potassium gluconate, 10 KCl, 10 Hepes, 2 NaATP, 0.2 NaGTP, 0.5 EGTA, pH 7.4, osmolarity 280 mosmol l^{-1}. Drug solutions were applied to cells by local perfusion through a capillary tube (1.1 mm inner diameter) positioned near the cell of interest (complete solution exchange occurs within 100 ms). The drug was then washed out very fast by local perfusion of wash solution. The solution flow was driven by gravity (flow rate ~1–5 ml min^{-1}) and controlled by miniature solenoid valves (The Lee Company, Westbrook, CT, USA). The external bath solution contained (mM): 150 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 Hepes, 10 glucose pH 7.4, osmolarity 310 mosmol l^{-1}. All recordings were done at –70 mV holding potential. Signals were recorded with the Axopatch-1C amplifier (Axon Instruments, Union City, CA, USA), digitized with Clampex 9.2 (Axon Instruments), and analysed using Mini Analysis Program (Synaptosoft, www.synaptosoft.com).

Stationary noise analysis

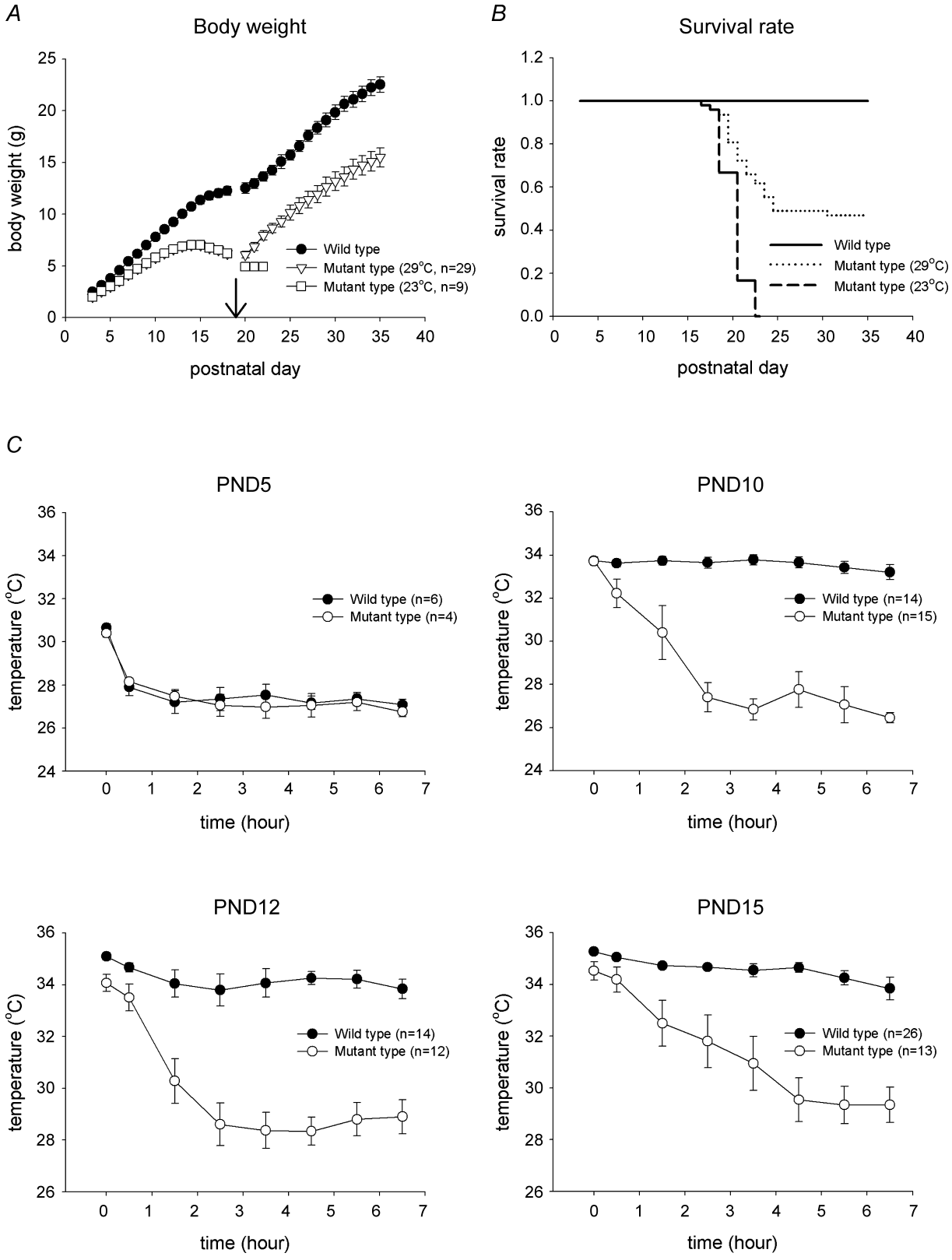
The goal of noise analysis was to obtain estimates of single-channel properties from the fluctuations about the macroscopic mean current (Traynelis & Wahl, 1997; Traynelis & Jaramillo, 1998). We used the stationary noise analysis of current responses to estimate the open probability at the peak, unitary current, and total number of channels (N) for nicotinic receptors using Channel Laboratory program (Synaptosoft). Nicotinic receptor currents were recorded and sampled at 10 kHz and analysed off-line. Each trace was highpass filtered at 10 Hz and analysed from the time of peak to the time of return to the baseline. Then the variance *versus* amplitude plot was generated. For a cell containing N channels that each pass a current of amplitude i when open, the mean current (I) is $I = iNP_{open}$, and the variance (σ^2) is $\sigma^2 = i^2NP_{open}(1 - P_{open})$, where P_{open} is the probability that the channel is open at the peak. Combining these two equations and substituting for P_{open} yield $\sigma^2 = iI - (I^2/N)$. This equation suggests, as has been found experimentally, that a plot of the current variance *versus* current amplitude is parabolic. By fitting the variance *versus* amplitude plot to the equation for each recording, we were able to obtain the estimated values of P_{open}, i and N.

Determination of UCP1 and β_3-adrenergic receptor mRNA level

cDNA probes for mouse UCP1 and β_3-adrenergic receptor were prepared by RT-PCR by use of first-strand cDNA from mouse interscapular BAT (Cinti, 2000) total RNA. For Northern blot analysis, mice were killed under intraperitoneal anaesthetic by an injection with an overdose of Avertin [2% tribromoethanol/tertiaryamylalcohol (Sigma-Aldrich, St. Louis, MO, USA)]. Interscapular BAT was quickly removed and cleaned of the surrounding tissues under a dissecting microscope, and frozen and stored at −70°C until RNA extraction. The PCR primers used to generate these probes were as follows (Cancello *et al.* 1998). UCP1: 5′primer, 5′-CCTTTTGGTCTCTGCCCTCCGAGCC-3′; and 3′primer, 5′-GCATAGGAGCCCAGCATAGGAGCCC-3′. β_3-Adrenergic receptor: 5′primer, 5′-CTGCTAGCAT-CGAGACCTT-3′; and 3′primer, 5′-CGAGCATAGA-CGAAGAGCAT-3′. cDNA probes were radiolabelled with [α-^{32}P]dCTP by the random labelling method. A 10 μg quantity of total RNA from the interscapular BAT was separated on a 1.5% agarose gel. The filters were hybridized with ^{32}P-labelled probe (10^6 c.p.m. ml^{-1}) for 18 h at 65°C in hybridization solution containing 9.9% PEG8000, 7% SDS, 0.01 M EDTA (pH 7.6), 0.25 M NaCl and 0.085 M $NaHPO_4$ (pH 7.2). Densitometric analysis of hybridization intensity was accomplished using Molecular Analyst software (Bio-Rad, Hercules, CA, USA) on an IBM computer.

Data analysis

A two tailed t test was used for statistical analysis. Mann-Whitney U test was applied in the analysis of body temperatures. Electrophysiology data were analysed



using Mini Analysis Program (Synaptosoft). All values are expressed as means ± standard error of the mean.

Results

Cold sensitivity of AChE mutant mice

The AChE-deficient mice were indistinguishable from their littermates at birth. However, they exhibited retardation in weight gain, which became noticeable at around PND15–17, and they eventually died soon after weaning when maintained at room temperature (23°C) without receiving any special care (Fig. 1*A*). In the previous report about AChE knockout mice by Duysen *et al.* (2002), the cause of death was proposed to be due to both impaired food intake and a defect in thermoregulation (Duysen *et al.* 2002). In that report, the authors suggested that the inability of AChE mutant mice to eat solid food was due to their weak muscles (Duysen *et al.* 2002). However, we found no difference in food intake of solid pellets between AChE mutant mice and their wild-type littermates when maintained at 29°C (Supplemental Fig. 2). This difference in phenotype could be due to a difference in genetic background between our knockout mice and those reported by Duysen *et al.* (2002) There have been many reports that the phenotype of same-gene knockout mice can be different depending on the genetic background (Matthias *et al.* 1999; Hofmann *et al.* 2001). We found that the primary cause of death of the mutant mice reared at 23°C was impaired thermoregulation because the mutants reared at thermoneutral temperature (about 29°C) without any change in diet survived at a much higher rate compared with the mice reared at 23°C, and they began to resume gaining weight at a rate comparable to that of wild-type mice (Fig. 1*A*). Thus, AChE-deficient mice that were reared at thermoneutral temperature showed an increased survival rate of about 50% at PND35 compared with 0% at 23°C (Fig. 1*B*).

When kept at 23°C in isolation from the mother, AChE-deficient mice were easily distinguishable from their wild-type littermates, not only by their small body size, but also by their severe shivering behaviour. However, when the AChE mutant mice were transferred to 29°C, this shivering behaviour was no longer observed, suggesting that the shivering behaviour of the mutant mice was a response to coldness, and presumably reflected an attempt of the mutant mice to generate heat through shivering thermogenesis. These results raised the possibility that the mutant mice might have a difficulty in maintaining body temperature by non-shivering thermogenesis alone.

To see if the mutant mice had a problem in maintaining body temperature, we measured the surface body temperature of the animals while they were exposed to 23°C in isolation from their mother. At PND5, both wild-type and mutant mice were equally unable to maintain their body temperature (Fig. 1*C*). However, at PND10 and thereafter, wild-type mice could maintain their body temperature consistently at least for 6 h under the conditions employed, whereas the body temperature of the AChE-deficient mice dropped rapidly within 1 h under the same conditions (Fig. 1*C*). At PND12 and 15, the body temperatures of the mutants were slightly yet significantly lower than those of the wild-type mice (Fig. 1*C*, the time point 0 min, two-tailed *t* test; $P = 0.006$ at PND 12 and $P = 0.008$ at PND 15) even when they were kept in the home cage with the mother, suggesting that the mutation may have caused a chronic and severe effect in the affected animals. Therefore, to avoid the potential chronic effect of the mutation, we used PND10 pups of the mice in further experiments to examine the mechanism of the hypothermia induced by a defect in AChE.

Induction of hypothermia by an alteration of SG cholinergic signalling

In adult mice it has been shown that exposure to AChE inhibitors elicits hypothermia by increasing muscarinic signalling in the hypothalamus (Clement, 1993). Thus, such hypothermia is blocked by atropine sulphate, which is a widely used muscarinic receptor antagonist that can cross the blood–brain barrier (BBB), but it is not blocked by methyl-atropine, which cannot cross the BBB (Clement, 1993; Sanchez & Meier, 1993), indicating that the site of action is in the central nervous system (CNS). There has been no previous report on infant mice. To test whether alterations of cholinergic signalling could induce hypothermia in infant mice and to determine the site of action, the wild-type PND10 and adult mice were exposed to the muscarinic agonists oxotremorine, which can cross the BBB, and oxotremorine-M, which cannot cross the BBB (Sethy & Francis, 1990; Sanchez & Meier, 1993). As previously reported, only oxotremorine, but not oxotremorine-M, was able to induce a robust hypothermia in adult mice (Fig. 2*A*, right). In contrast to adult mice, oxotremorine-M as well as oxotremorine induced a robust hypothermia in PND10 wild-type mice.

Figure 1. Growth retardation and hypothermia in acetylcholinesterase (AChE) mutant mice
A, comparison of the body weight between wild-type (●) and AChE mutant mice (□, 23°C; ▽, 29°C).The arrow indicates the weaning day (postnatal day (PND) 19). *B*, survival rate of wild-type mice (continuous line), and AChE mutant mice reared at room temperature (23 ± 1°C; dashed line) and thermoneutral temperature (29 ± 1°C; dotted line). *C*, time course of surface body temperature in wild-type and AChE mutant mice isolated from the parental cage at the indicated age, and then left at room temperature (23 ± 1°C).

These results indicate that the muscarinic receptor agonist induces hypothermia in infant mice, and suggest that the site of action in infant mice is at the periphery, presumably at the SG.

To see if the hypothermia in PND10 mutant pups is sensitive to muscarinic blockers as in organophosphate-induced hypothermia, we administered methyl-atropine ($2\ mg\ kg^{-1}$), a BBB-impermeable muscarinic receptor antagonist (Clement, 1993), into the mutant mice and found that the hypothermia was partially rescued (Fig. 2*B*). Similar to methyl-atropine, administration of atropine sulphate ($2\ mg\ kg^{-1}$), a BBB-permeable muscarinic receptor antagonist (Clement, 1993), caused a partial rescue in PND10 mutant pups (data not shown). These results suggest that peripheral muscarinic receptors that are constitutively activated by prolonged exposure to increased ACh may be responsible for the induction of hypothermia in the mutant pups.

Decreased nicotinic signalling by prolonged activation of muscarinic receptors and nicotinic receptors in the SG of AChE mutant pups

Since the primary site of peripheral cholinergic signalling related to thermogenesis is at the SG, we recorded whole-cell currents induced by nicotine ($60\ \mu M$), a nicotinic receptor agonist, in acutely isolated SG neurons from PND10 pups to clarify the role of nicotinic receptors in the induction of hypothermia in the mutant mice. Nicotine application induced large inward currents (2249.1 ± 135.1 pA, $n = 13$), with a rapid onset in acutely isolated SG neurons of wild-type mice (Fig. 3*A*). Nicotinic receptor currents were significantly reduced by about 40% in AChE-deficient mice (1322.2 ± 105.2 pA, $n = 19$, $P < 0.001$) compared with the wild-type mice (Fig. 3*B*).

The results of the methyl-atropine injection study (Fig. 2*B*) revealed that the prolonged activation of muscarinic receptors was responsible for the hypothermia in the mutants. Therefore, we investigated whether prolonged stimulation of muscarinic receptors had an effect on nicotinic receptor currents in the wild-type SG neurons. After pretreatment with $10\ \mu M$ oxotremorine-M, a selective muscarinic receptor agonist, for 1 h, nicotinic receptor currents were recorded in the presence of $10\ \mu M$ oxotremorine-M (Fig. 3*C*). The amplitude of nicotinic receptor currents was decreased by about 40% after the exposure to oxotremorine-M (Fig. 3*C* and *E*).

The accumulation of ACh in the synaptic cleft resulting from the lack of AChE can chronically affect nicotinic receptors as well as muscarinic receptors. To mimic the effect of a long-term exposure of the nicotinic receptor to ACh, we recorded nicotinic receptor currents in the wild-type SG neurons after a prolonged nicotine ($1\ \mu M$) treatment for 1 h. We observed that the nicotinic receptor currents were reduced by 40% by long-term treatment with a low concentration of nicotine (Fig. 3*D* and *E*). These results suggest that a possible cause of hypothermia in the AChE mutant mice might be due to a reduction of the nicotinic receptor currents by prolonged activation of both nicotinic and muscarinic receptors in the SG neurons.

The reduction in nicotinic receptor currents can be explained by either a change of receptor property by the change of subtype composition, or removal of the

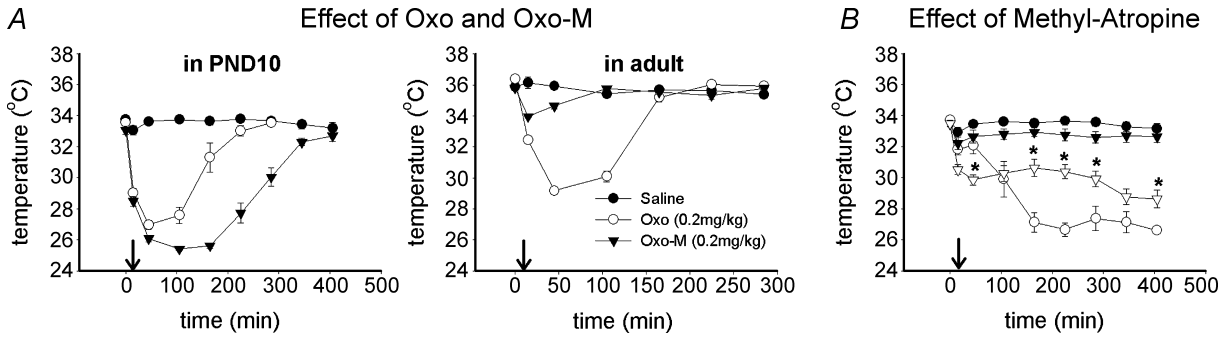


Figure 2. Partial rescue of hypothermia by blockage of muscarinic receptor in peripheral sympathetic ganglion (SG) neurons

A, time course of body temperature showing hypothermia in PND10 (left) and adult (right) wild-type mice administered oxotremorine (Oxo) or oxotremorine-M (Oxo-M). Saline ($0.01\ ml\ g^{-1}$; ●), Oxo ($0.2\ mg\ kg^{-1}$; ○) and Oxo-M ($0.2\ mg\ kg^{-1}$; ▼) were administered intraperitoneally. The arrow indicates the time of drug injection. *B*, time course of body temperature after methyl-atropine or saline injection. ●, Wild-type mice injected with saline ($n = 14$); ●, AChE mutant mice injected with saline ($n = 6$); ▼, wild-type mice injected with methyl-atropine ($2\ mg\ kg^{-1}$, $n = 11$); ▽, AChE mutant mice injected with methyl-atropine ($2\ mg\ kg^{-1}$, $n = 9$). *Significant difference ($P < 0.05$) between the AChE mutant group injected with methyl-atropine and the group injected with saline (Mann-Whitney *U* test).

receptor channels from the cell surface. To investigate the property of nicotinic receptor in SG neurons of the mutant mice, we first measured the current evoked by different concentrations of ACh and then calculated a half-maximal concentration (EC_{50}) for ACh in wild-type and mutant mice. As shown in Fig. 4, EC_{50} values for ACh were not significantly different between wild-type (14.9 μM) and mutant mice (17.3 μM). We also compared the desensitization kinetics by measuring and comparing the steady-state-to-peak ratios of the nicotinic receptor currents in wild-type and mutant SG neurons, and found no significant difference (data not shown). To estimate the single-channel properties in more detail, we recorded nicotine-induced whole-cell currents under a stationary condition (Fig. 5*A*) and performed stationary noise analysis (Fig. 5*B*; Traynelis & Wahl, 1997; Traynelis & Jaramillo, 1998). The results of the analysis showed that the estimates of the unitary current and open probability at the peak were not different between the wild-type and mutant mice (Fig. 5*C* and *D*). These results indicate that the single channel properties were not changed and suggest that the reduction of nicotinic receptor current is probably due to a reduced total number of channels in mutant SG neurons. These results are consistent with the idea that the causes of the reduction of nicotinic receptor currents in AChE mutant mice include the removal of nicotinic receptor channels from the surface by the prolonged exposure of ACh in the synaptic cleft.

Full rescue of the hypothermia in AChE mutant mice by administration of a potentiator of nicotinic receptors

As shown in Fig. 2*B*, the body temperature of AChE mutant pups was not fully recovered by the injection of methyl-atropine. We hypothesized that if this incomplete rescue was due to the reduced nicotinic currents in the mutants, the residual hypothermia exhibited in AChE mutant pups in the presence of atropine should be fully rescued by potentiating the nicotinic receptor function. To test this hypothesis, we used ivermectin, which acts as a positive allosteric effector for the α7-subunit-containing nicotinic receptors and thus strongly potentiates ACh-evoked currents (Krause *et al.* 1998). Ivermectin has been shown not to cross the BBB because it is pumped out by P-glycoprotein (Van Asperen *et al.* 1997; Jun *et al.* 2000), conveniently limiting the effect to the periphery when injected intraperitoneally. As shown in Fig. 6*C* and *D*, the body temperatures of PND10 AChE mutant mice administered with both ivermectin (3 mg kg^{-1}) and methyl-atropine (2 mg kg^{-1}) were almost completely rescued to the level of wild-type mice. Furthermore, ivermectin potentiated ACh-induced currents by 43.1 ± 5.0% ($n = 5$) in SG

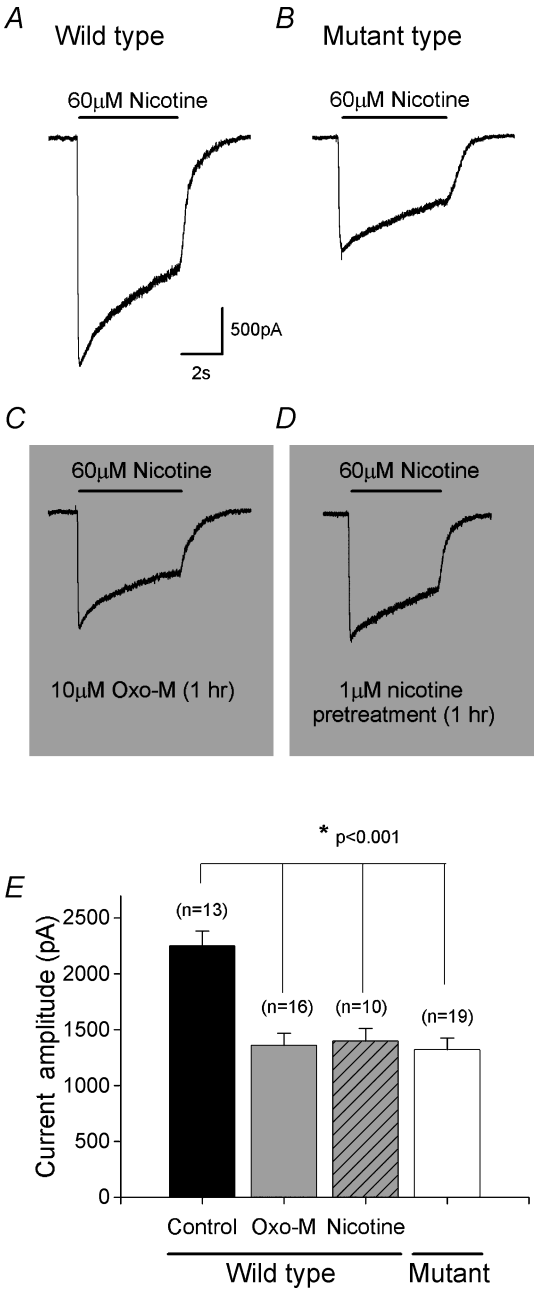


Figure 3. Decreased nicotine-induced current in AChE mutant SG neurons
A and *B*, nicotinic receptor currents were recorded under whole-cell patch configuration at −70 mV holding potential in SG neurons from wild-type and AChE mutant mice. Nicotine (60 μM) was applied to cells for 5 s by local perfusion through a capillary tube positioned near the cell of interest (complete solution exchange occurs within 100 ms). The drug was the washed out very quickly by local perfusion of the wash solution. *C*, after pretreatment with 10 μM oxotremorine-M for 1 h, nicotinic receptor currents were recorded in wild-type SG neurons in 10 μM oxotremorine-M-containing solution. *D*, after pretreatment with 1 μM nicotine for 1 h, we recorded nicotinic receptor current in wild-type SG neurons in normal solution. *E*, average current amplitude. The average amplitude of current in the mutant, and oxo-M- and nicotine-treated neurons, was compared with that of the wild-type control (*$P < 0.001$, two-tailed t test).

neurons from AChE mutant mice (Fig. 6*A* and *B*), indicating that SG neurons express a significant level of α7-subunit-containing nicotinic receptors. These results suggest that the primary underlying cause of hypothermia in AChE mutant mice is a downregulation of the nicotinic receptor activity in the SG neurons.

Chronic effects of impaired cholinergic signalling: depletion of white adipose tissue and premature death

SG neurons, innervating BAT, release noradrenaline to activate β_3-adrenergic receptors on the brown adipocytes; the β_3-adrenergic receptor is the major receptor involved in thermogenesis (Thomas & Palmiter, 1997; Zhao *et al.* 1998). To see if the hypothermia observed in PND10 mutant mice is due to an impairment of heat generation in BAT, we examined the animals for any changes in the downstream signalling components that are known to be important in thermogenesis in BAT. We measured the expression level of mRNA for β_3-adrenergic receptors in the BAT of the animals by the Northern blot analysis. The level of β_3-adrenergic receptor mRNA in BAT was similar between the two genotypes at PND10, but was significantly decreased at PND12 in the AChE-deficient mice (Fig. 7*A*). Next, UCP1 mRNA level, a hallmark of the thermogenic recruitment process (Enerback *et al.* 1997; Kozak & Harper, 2000), was assessed by the Northern blot analysis. Up to PND10, the UCP1 mRNA level of the AChE mutant BAT was not significantly different from that of the wild type, but thereafter it rapidly decreased compared with the wild type (Fig. 7*B*). Taken together, these results indicate that the primary cause of hypothermia in the PND10 mutant mice is most likely to be a downregulation of nicotinic receptors in SG neurons, rather than a defect in the downstream components of BAT. Consistently, the relative amount of BAT per body weight in the mutant mice was not significantly different from the wild type when measured up to PND15 (Fig. 7*C*).

The rapid decrease in the mRNA expression levels of β_3-adrenergic receptors and UCP1 at PND 12 and beyond, suggests a chronic effect of the impaired cholinergic signalling in SG neurons. The severe shivering behaviour of infant mutant mice predicts an overconsumption of energy source from white adipose tissue. In fact, we observed a significant reduction in the ratio of white adipose tissue to body weight in PND15 mutant mice (Fig. 7*D*) and an almost complete depletion of white adipose tissue in PND 20 mutant mice reared at 23°C, as confirmed by the nuclear magnetic resonance image (Fig. 7*E*). The loss of white adipose tissue and the reduction in body weight coincide with a premature death before PND23 of mutant mice reared at 23°C (Fig. 1*B*).

Discussion

In this study, we have demonstrated the mechanism of hypothermia in infant mice that are deficient in AChE. In contrast to previous studies using adult mice, here we report that the peripheral nervous system is critical in the thermoregulation in infant mice. The hypothermic phenotype of the infant AChE mutant mice was similar to the hypothermia observed in animals exposed to pesticides or nerve gases containing organophosphate, a potent AChE inhibitor (Clement, 1991*a*,*b*, 1993; Gordon & Grantham, 1999). In previous studies with adult rats or mice, it has been suggested that hypothermia induced by exposure to AChE inhibitors, or agonists for muscarinic

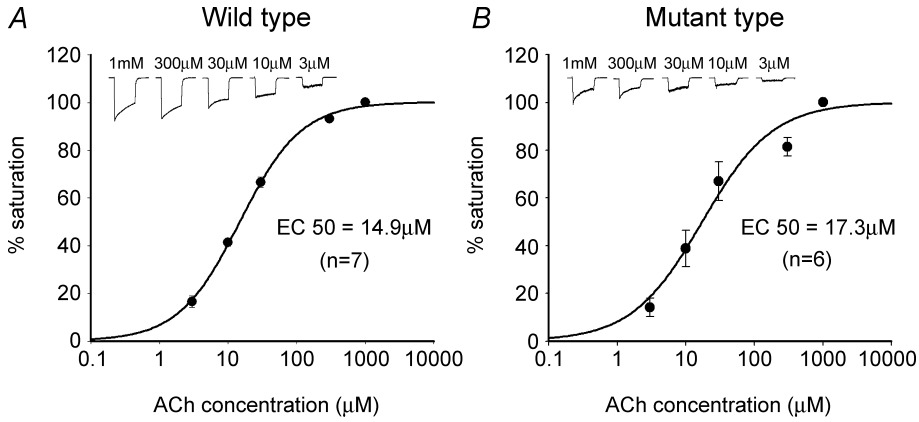


Figure 4. Dose–response curves to ACh in SG neurons of wild-type and AChE mutant mice
Dose–response curves for the peak currents induced by ACh in SG neurons of wild-type (*A*) and AChE mutant mice (*B*). Individual cells were exposed to varying ACh concentration (3 μM, 10 μM, 30 μM, 300 μM and 1 mM). The currents recorded from each cell were normalized relative to the current induced by 1 mM Ach, and the dose–response relationship was plotted. The EC_{50} was calculated by fitting the dose–response relationship to the logistic function. The vertical error bars indicate the standard error of the mean.

receptors, might be due to a lowering of the set point for heat release in the hypothalamus by increasing cholinergic signalling. This hypothermia was blocked by atropine sulphate, a widely used muscarinic receptor antagonist that can cross the BBB, but not by methyl-atropine, which cannot cross the barrier (Clement, 1991*a*, 1993; Sanchez & Meier, 1993). Our results are consistent with those previous reports in that the wild-type adult mice showed hypothermia when injected with oxotremorine, which can cross the BBB, but failed to show hypothermia when injected with oxotremorine-M, which is unable to cross the BBB (Fig. 2*A*). However, the same oxotremorine-M induced a robust hypothermia in PND10 wild-type mice (Fig. 2*A*), suggesting that unlike in adults the peripheral

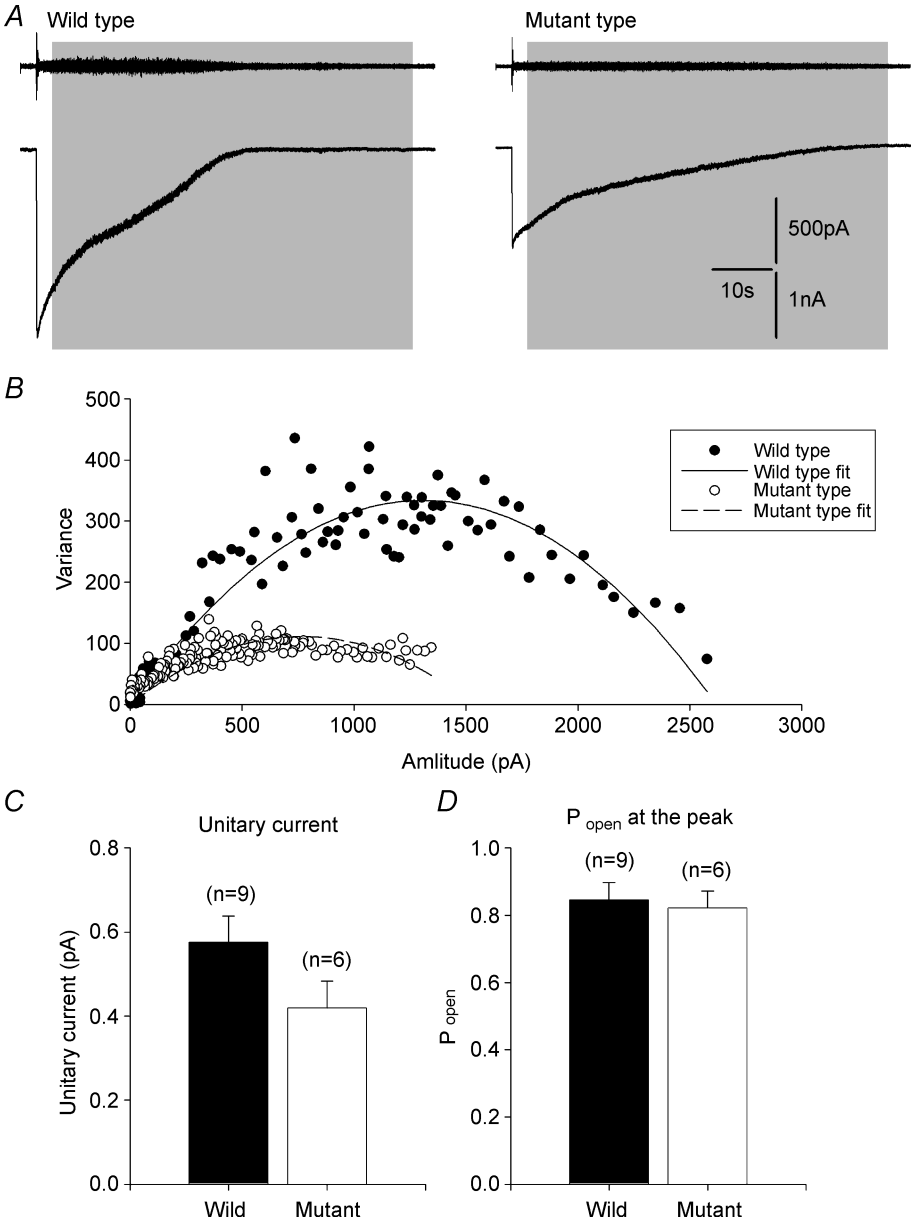


Figure 5. Stationary noise analysis in nicotinic receptor current from wild-type and AChE mutant mice
A, nicotinic receptor currents (bottom) were high-pass filtered at 10 Hz (top). Grey boxes indicate the regions used for noise analysis. *B*, plot of variance *versus* amplitude for wild-type (●) and mutant (○). The two curves represent the fitted parabola for wild-type (continuous line) and the mutant (dashed line). *C*, the estimated unitary current was obtained by noise analysis. There was no significant difference between the wild-type and mutant (unpaired *t* test, $P = 0.12$). *D*, the estimated open probability at the peak (P_{open}) was measured by noise analysis. There was no significant difference between the wild-type and mutant (unpaired *t* test, $P = 0.76$). See Methods for detailed information on the algorithm.

muscarinic receptors are critical in the thermogenesis of PND10 mice. Consistently, the hypothermia observed in PND10 AChE mutant mice was partially recovered by an injection of methyl-atropine which was shown to be ineffective in adult mice intoxicated by AChE inhibitors or muscarinic agonists due to its impermeability to the BBB (Fig. 2*B*). The hypothermia observed in PND10 AChE mutant mice was fully recovered by a coinjection with methyl-atropine and ivermectin, which cannot cross the BBB (Fig. 6*C* and *D*). Infants are known to be more dependent on peripheral thermogenesis than adults in response to cold (Blackburn, 2003). Therefore, our results support the idea that the peripheral cholinergic signalling is essential in thermogenesis in PND10 mice.

The peripheral cholinergic signalling is directly involved in the sympathetic as well as the parasympathetic nervous system. SG neurons are known to innervate BAT, which is the major tissue for heat generation in response to cold in small animals such as rodents and infants, and activate several thermogenic effectors, such as skin, to cause cutaneous vasoconstriction to protect from cold.

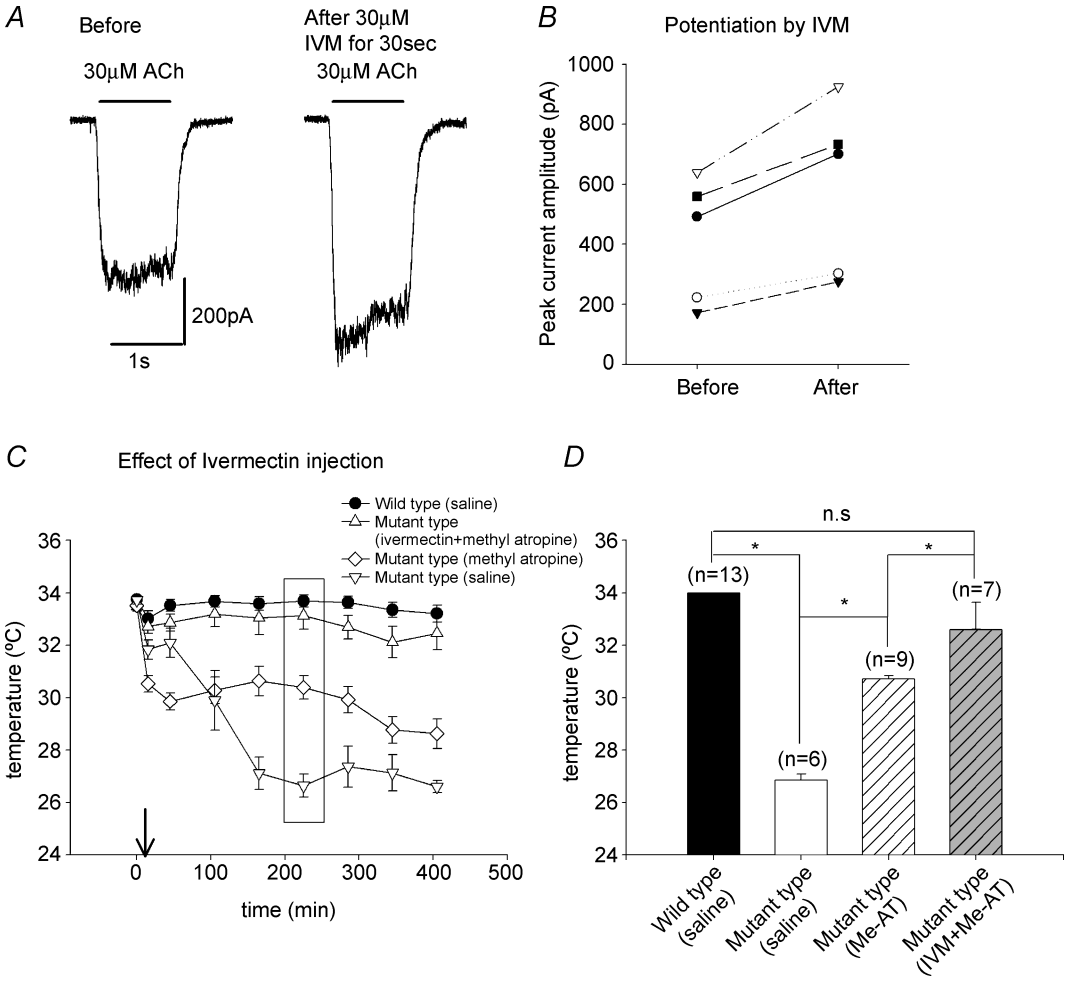


Figure 6. Full rescue of hypothermia by ivermectin in AChE mutant mice
A, whole-cell current recordings of 30 μM ACh application before and after a treatment with 30 μM ivermectin for 30 s in SG neurons from AChE mutant pups. IVM, ivermectin. *B*, changes in peak current amplitude before and after IVM treatment in each neuron are represented by different symbols. *C*, effect of IVM injection on the time course of body temperature of PND10 animals. ●, Wild type injected with saline; △, AChE mutant mice with IVM (3 mg kg^{-1}) and methyl-atropine (2 mg kg^{-1}); ◇, AChE mutant mice with methyl-atropine (2 mg kg^{-1}); ▽, AChE mutant mice with saline. The vertical box represents snapshot averages of body temperature as shown in *D*. *D*, average body temperatures at 225 min after injection of each drug. There was no significant difference between wild type injected with saline and mutant type injected with methyl-atropine and IVM (Mann-Whitney *U* test, $P = 0.633$). *Significant difference ($P < 0.005$, Mann-Whitney *U* test).

Therefore, the hypothermia displayed in infant AChE mutant mice was most probably due to an alteration of cholinergic signalling at the level of the SG. Surprisingly, we found that the alteration of cholinergic signalling in mutant SG neurons is a direct result of a reduction in nicotinic receptor currents (Fig. 3*A*, *B* and *E*). This reduction of nicotinic receptor currents in the mutant mice is expected to result in a decrease in the excitation of the SG neurons, which in turn will decrease the major excitatory inputs to BAT and other peripheral thermogenic effectors. This explanation can provide an answer to the paradox that an enhancement of cholinergic signalling due to a deficiency of AChE, either by a mutation or by an exposure to AChE inhibitors, such as nerve gases or pesticides, induces hypothermia instead of hyperthermia.

Our results showed that the unexpected reduction in nicotinic receptor currents in AChE mutant pups is a result of prolonged activation of muscarinic and nicotinic receptors. We were able to mimic the reduction of nicotinic receptor currents in mutant mice by long-term treatment with agonists for muscarinic and nicotinic receptors in wild-type SG neurons (Fig. 3*C*, *D* and *E*). Consistent

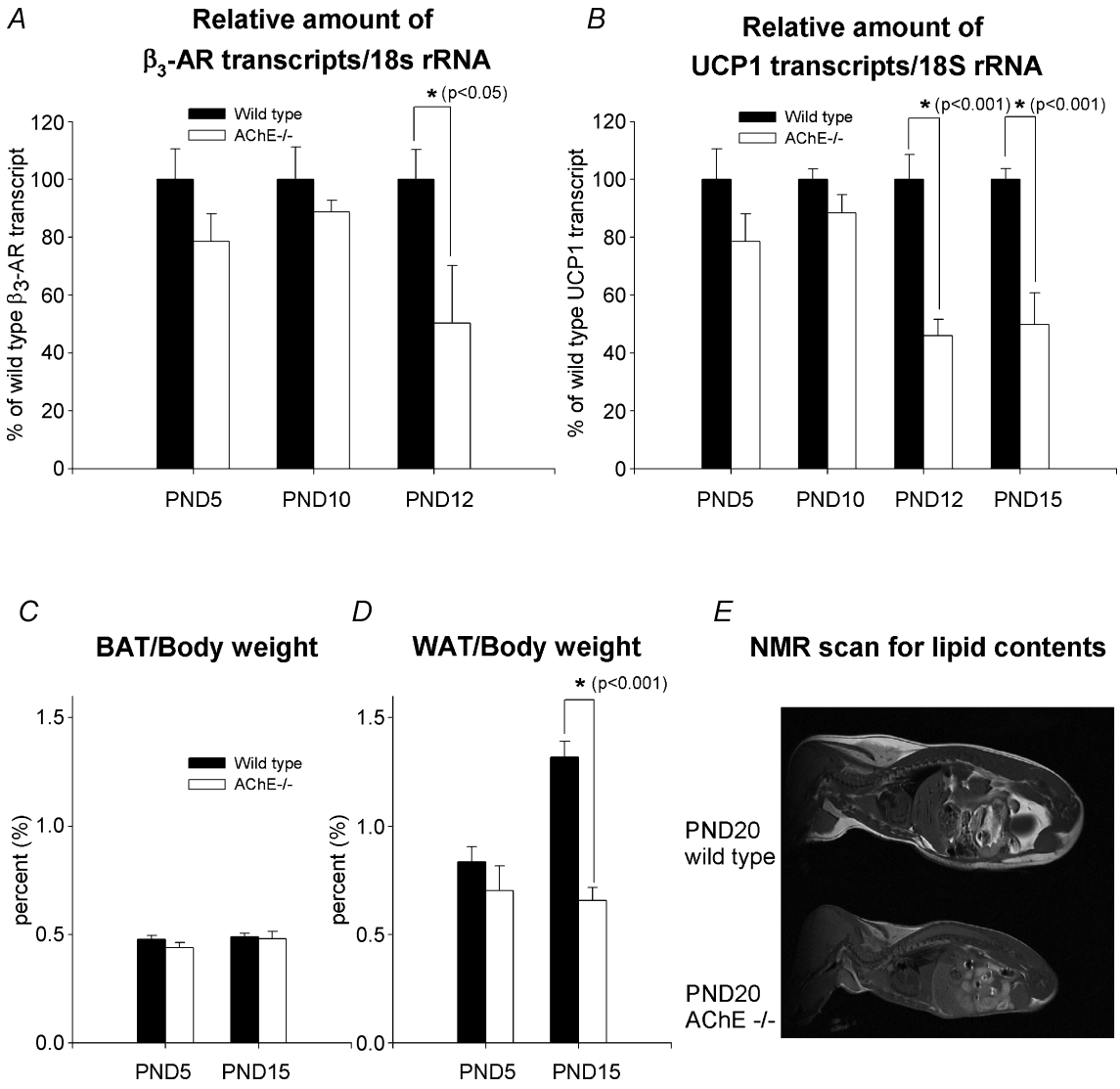


Figure 7. Decreased white adipose tissue (WAT) and premature death in AChE mutant mice
A and *B*, the mRNA expression levels of β_3-adrenergic receptor and UCP1 were verified using the Northern blot analysis of total RNA from brown adipose tissue (BAT) of wild-type (filled bar) and AChE mutant mice (open bar) (PND12, β_3-adrenergic receptor mRNA level, two-tailed *t* test, $^*P < 0.05$; PND 12 and 15, UCP1 mRNA level, two-tailed *t* test, $^*P < 0.001$). *C* and *D*, percentage of BAT weight and WAT weight to body weight for the wild-type and AChE mutant; two-tailed *t* test, $^*P < 0.001$. *E*, white fat tissue is visualized in white on digital pictures obtained by nuclear magnetic resonance topography. White fat tissue deposition is shown for the wild type (top) and the AChE mutant type (bottom) reared at 23°C.

with our results, it has been reported that activators of protein kinase C (PKC), a downstream component of muscarinic receptors, could induce a rapid desensitization of nicotinic receptor in SG neurons (Downing & Role, 1987). In addition, Ca^{2+} influx through nicotinic receptor channels has been shown to serve as a physiological trigger for regulation of desensitization of the channels either directly or indirectly via PKC activation (Quick & Lester, 2002). In an earlier study, the desensitization of nicotinic receptor could be observed as a decline in responsiveness of the muscle cell to ACh during a continuous exposure to ACh (Katz & Thesleff, 1957). These reports, along with our results, support the hypothesis that under prolonged activation of muscarinic and nicotinic receptors due to a lack of AChE, nicotinic receptors are rapidly down-regulated, leading to a decrease in the excitation of SG neurons.

The importance of nicotinic receptor in thermoregulation is highlighted by the fact that the coinjection of methyl-atropine and ivermectin led to a full recovery from hypothermia in AChE mutant pups (Fig. 6*C* and *D*). The partial recovery from hypothermia by methyl-atropine alone provides two important implications. First, it implies that the overactivation of muscarinic receptors inhibits the nicotinic receptor signalling, which is the major carrier of thermoregulatory signal. Our study provides clues to the mystery of how atropine can be an effective therapeutic agent for hypothermia in animals exposed to AChE inhibitors. The blocking of muscarinic receptors by methyl-atropine leads to a relief from down-regulation of nicotinic receptors, resulting in partial recovery of hypothermia in AChE mutant pups. Second, the partiality of the recovery by methyl-atropine alone suggests that the chronic effect of ACh accumulation is a permanent reduction of nicotinic receptors. This point is strengthened by the full recovery of hypothermia by the addition of ivermectin, a BBB-impermeable potentiator of nicotinic receptors (Fig. 6*C* and *D*). The results from the ivermectin experiments emphasize the importance of nicotinic receptors in mediating the thermoregulatory signal in SG neurons of infants.

The possible mechanism of the reduction in nicotinic receptor currents can be either a receptor desensitization or removal of receptor channels from the cell surface. Our results favour the removal of nicotinic receptor channels from the cell surface as the potential mechanism. There was no significant difference in the EC_{50} value for ACh (Fig. 4) and desensitizing kinetics of nicotinic receptor currents between wild-type and mutant SG neurons (data not shown). The results of stationary noise analysis showed that the estimated single-channel properties of nicotinic receptor, including open probability at the peak and unitary current, were not changed in mutant SG neurons (Fig. 5*C* and *D*). In support of our hypothesis, there have been reports that the number of surface nicotinic receptors in the muscle endplate of AChE knockout mice is decreased by increased receptor internalization in response to excess ACh in the synaptic cleft (Adler *et al.* 2004; Girard *et al.* 2005). Taken together, these results imply that in SG neurons of AChE mutant mice, the cause of the reduction of nicotinic receptor currents is probably due to a reduced total number of channels in the cell surface.

Muscarinic receptors may play an important role in preventing an overactivation of nicotinic receptors in SG neurons and thus preventing an overstimulation of the thermogenic effectors. Under normal conditions muscarinic receptor activation might be kept at a minimal level due to the hydrolysis of ACh by AChE in the synaptic cleft. During an increased activity and increased release of ACh, the muscarinic receptors are more likely to be activated and serve to downregulate the nicotinic receptors, effectively preventing the relay of the signal to the thermogenic effectors. However, under aberrant conditions where muscarinic receptors are overactivated by chronic exposure to ACh (as in the AChE mutant mice), the downregulation of the nicotinic receptors by muscarinic receptor activation results in a permanent reduction in nicotinic receptor function in SG, leading to severe hypothermia.

We observed that the chronic hypothermia caused by impaired signalling to the BAT in AChE mutant mice induces a severe shivering behaviour in an attempt to produce heat by consuming white adipose tissue that is normally reserved for growth. This was evident in the reduced white adipose tissue content in the mutant mice (Fig. 7*D*). In human infants, dysfunction of thermoregulation can lead to death (Blackburn, 2003; Simbruner *et al.* 2005) in a similar manner to that shown in AChE mutant mice by a reduction in body weight and eventual death (Fig. 1*A* and *B*).

The same mechanism of reduced nicotinic receptor function in SG leading to hypothermia can be applied to explain the weak grip strength phenotype reported in the AChE knockout mice (Duysen *et al.* 2002). The lack of AChE activity can lead to the downregulation of nicotinic receptor function similarly in the neuromuscular junction. This reduction in nicotinic receptor signalling in knockout mice is expected to result in decreased responses in muscle cells. Consistently, it has been demonstrated that the nicotinic receptors in neuromuscular junction of AChE mutant mice are decreased in number (Adler *et al.* 2004; Girard *et al.* 2005). Therefore, the reduced muscle responsiveness caused by an attenuated signalling to the muscle cells in AChE mutant mice manifests as a weakness of grip strength.

From this study, we conclude that the peripheral cholinergic system at the level of SG is an essential player in thermoregulation in infants. We propose that a better remedy for hypothermia in infants exposed to nerve gases or pesticides is BBB-impermeable methyl-atropine,

rather than atropine sulphate, which crosses the BBB. This method will minimize the potential side-effects of atropine in the CNS.

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Acknowledgements

This work was supported by the Chemoinformatics Program and the Center-of-Excellence program at the Korea Institute of Science and Technology. This work was also supported by the Korea Research Foundation KRF-2005-070-C00096 (C.J.L.). We thank Joon-oh Park for mouse care and Il-hwan Choi for video recording.

Supplemental material

The online version of this paper can be accessed at:
DOI: 10.1113/jphysiol.2006.120147
http://jp.physoc.org/cgi/content/full/jphysiol.2006.120147/DC1
and contains supplemental material consisting of two files entitled Generation of AChE mutant mice (containing Supplemental Figs 1 and 2) and Anatomy of the adipose organ.

This material can also be found as part of the full-text HTML version available from http://www.blackwell-synergy.com