Pituitary adenylate cyclase-activating polypeptide maintains neonatal breathing but not metabolism during mild reductions in ambient temperature

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Cummings KJ, Willie C, Wilson RJ. Pituitary adenylate cyclase-activating polypeptide maintains neonatal breathing but not metabolism during mild reductions in ambient temperature. Am J Physiol Regul Integr Comp Physiol 294: R956–R965, 2008. First published January 9, 2007; doi:10.1152/ajpregu.00637.2007.—Mild reductions in ambient temperature dramatically increase the mortality of neonatal mice deficient in pituitary adenylate cyclase-activating polypeptide (PACAP), with the majority of animals succumbing in the second postnatal week. During anesthesia-induced hypothermia, PACAP−/− mice at this age are also vulnerable to prolonged apneas and sudden death. From these observations, we hypothesized that before the onset of genotype-specific mortality and in the absence of anesthetic, the breathing of PACAP-deficient mice is more susceptible to mild reductions in ambient temperature than wild-type littersates. To test this hypothesis, we recorded breathing in one group of postnatal day 4 PACAP+/+, +/−, and −/− neonates (using unrestrained, flow-through plethysmography) and metabolic rate in a separate group (using indirect calorimetry), both of which were exposed acutely to ambient temperatures slightly below (29°C), slightly above (36°C), or at thermoneutrality (32°C). At 32°C, the breathing frequency of PACAP−/− neonates was significantly less than PACAP+/+ littersates. Reducing the ambient temperature to 29°C caused a significant suppression of tidal volume and ventilation in both PACAP+/+ and +/− animals, while the tidal volume and ventilation of PACAP+/+ animals remained unchanged. Genotype had no effect on the ventilatory responses to ambient warming. At all three ambient temperatures, genotype had no influence on oxygen consumption or body temperature. These results suggest that during mild reductions in ambient temperature, PACAP is vital for the preservation of neonatal tidal volume and ventilation, but not for metabolic rate or body temperature.

neonatal apnea; temperature; metabolism; hypoxia; neonatal apnea; sudden infant death syndrome

THE PRIMARY ROLE OF THE RESPIRATORY control system is to couple ventilation (VE) to changes in metabolic rate for the maintenance of blood gases. In small neonatal rodents, a drop in ambient temperature just outside the thermoneutral zone initiates nonshivering thermogenesis and an increase in oxygen consumption (VO2). Despite this metabolic response, the body temperature of small neonatal rodents continues to fall with ambient temperature, owing to their large surface area-to-volume ratio (28). This causes a potential problem for the neonatal respiratory control system; a fall in body temperature has a suppressive effect on neuronal function, including those involved in the control of breathing [Q10 effect: (27, 36, 41)]. Neonates must therefore have compensatory mechanisms to mitigate the Q10 effect and maintain the tight coupling between VE and metabolic rate.

Pituitary adenylate cyclase-activating polypeptide (PACAP) has potent modulatory effects on both the central and peripheral nervous systems. The primary structure of PACAP is remarkably constrained throughout evolution, suggesting one or more critical developmental and/or physiological roles (38). PACAP stimulates three classes of G protein-coupled receptors that utilize cAMP and phosphoinositol species to produce its cellular effects (42). At the system level, PACAP has been implicated in the proliferation, differentiation, and survival of neural progenitor cells within the central nervous system (30, 31, 43). However, PACAP is also involved in a range of acute physiological processes with mounting evidence suggesting PACAP may be vital for maintaining homeostasis in the face of thermal and metabolic stress (1, 39); a large proportion of PACAP−/− mice do not survive the neonatal period when raised in slightly cooler environments (12), and PACAP-deficient adult mice have impaired catecholaminergic counterregulatory responses to hypoglycemia (13) and hypotension (22).

Previously, we showed that postnatal day 4 (P4) PACAP-deficient neonates have blunted respiratory responses to hypoxia and hypercapnia. Furthermore, in slightly older (P7–P14) PACAP−/− neonates, anesthetic-induced hypothermia causes long-duration apneas that culminate in atrioventricular block (8). Based on these observations, PACAP might also be important for maintaining VE in unanesthetized animals during small drops in ambient temperature that are similar to those normally experienced in the litter. However, given the abundance of PACAP within the hypothalamus (2, 34), the inability of older PACAP−/− neonatal mice to maintain body temperature (12), and the suppressive effects of anesthetic on metabolism, it is equally possible that the breathing phenotypes that we previously demonstrated in neonatal PACAP−/− mice are secondary to a suppression of metabolic rate.

Here, we test the hypothesis that PACAP is important for the preservation of VE, but not metabolic rate, during small reductions in ambient temperature. Our results indicate that mild ambient cooling is an environmental stressor that selectively inhibits the breathing of PACAP-deficient P4 neonates, an effect that is not related to abnormal responses of metabolic rate or body temperature.

MATERIALS AND METHODS

Experimental procedures were approved by the University of Calgary Animal Care Committee and were in accordance with national guidelines. A total of 225 neonates were used in this study.
Animals

Mice were housed at 22–24°C with a 12:12-h light-dark cycle and were given food and water ad libitum. PACAP$^{+/+}$, $^{+/-}$, and $^{-/-}$ mice were bred from PACAP$^{+/+}$ mice kept on a 129SvJ~C57BL/6 hybrid background. This is the same mixed line used in previous studies (8, 11, 12). Although there is some between-strain variability in terms of phenotype severity, previous studies have suggested that high mortality in PACAP knockout neonatal mice is strain independent (11, 13, 14, 39). Littermates were used in all experiments. Genotyping of mice was performed after data were analyzed; thus, experiments and analysis were performed in a blinded fashion.

Plethysmography

Whole body, continuous flow, unrestrained plethysmography was performed as described previously (8). Prior to experimentation, animals were kept at an ambient temperature of 32 ± 0.5°C, within the thermoneutral range of animals of this age (4, 29), and close to the ambient temperature within the litter (29). Two 20-ml chambers, one acting as a reference chamber, were used to detect the pressure signal generated from breathing in neonatal mice weighing <3g [tidal volume (VT) 0.05 ml (27)]. Ambient temperature was monitored continuously using a thermocouple inserted through the top of the chamber, which was checked periodically against a standard mercury thermometer (Omega Engineering, Stamford, CT). The experimental protocol is shown in Fig. 1A. Animals were placed into the animal chamber, held at 32 ± 0.5°C, and allowed a 5-min settle period. Breathing was then recorded for 5 min after which the temperature was either maintained at 32°C (thermoneutral control, $n = 36$), cooled to 29 ± 0.5°C (cool protocol, $n = 54$), or warmed to 36 ± 0.5°C (warm protocol, $n = 45$) by adjusting the position of the chamber relative to a heat lamp. The rate of cooling and heating were ~0.6°C/min and 0.4°C/min, respectively. Recordings were continued throughout the period of temperature change (~5 min for cooling, 10 min for warming), and at the new steady-state temperature. The total time of each protocol was 20 min. However, the rate of warming was slower than the rate of cooling. Consequently, it took more of the protocol to reach the steady-state warm temperature (36°C) than the steady-state cool temperature (29°C), and thus, when comparing the two protocols, please note that the duration at 36°C was slightly shorter than the

![Fig. 1. Methodology for assessing breathing and metabolic rate in pituitary adenylate cyclase-activating polypeptide (PACAP) genotypes. A: three plethysmograph protocols were used: 1) warm, 2) thermoneutral control, 3) cool. Ta, ambient temperature. B: representative recording of changes in fractional inspired O2 (FIO2). C: mass of PACAP genotypes used in plethysmography and metabolism experiments. Note that the mass of PACAP$^{+/-}$ postnatal day 4 (P4) neonates is not statistically different than that of wild-type littermates, while that of PACAP$^{-/-}$ neonates is significantly lower than that of both PACAP$^{+/-}$ and $^{+/-}$ littermates (1-factor ANOVA: $P < 0.001$; *Tukey post hoc PACAP$^{+/-}$ vs. PACAP$^{-/-}$: $P < 0.001$; Tukey post hoc PACAP$^{+/-}$ vs. PACAP$^{+/-}$: $P = 0.700$).](http://ajpregu.physiology.org/https://ajpregu.physiology.org/10.1152/ajpregu.00970.2007)
duration at 29°C. The chamber was flushed with a continuous flow of room air, regulated by a pair of precision gas flow controllers, at a rate sufficient to exchange the air within the chamber in <1 min.

Data Analysis

Of the 20 min of breathing recorded, three 2-min segments were used for analysis (Fig. 1A): baseline (minutes 4 and 5), during temperature change (minutes 7 and 8), and new steady-state temperature (minutes 19 and 20). Analysis was performed offline on a total of 123,973 breaths using semiautomated analysis software written in VEE (by R. J. A. Wilson; Agilent Technologies, Palo Alto, CA), as described previously (8). Parameters derived from the pressure waveform associated with an animal breathing within a barometric chamber were rate (breaths/min), index of VT, and index of VE. In addition, the standard deviation of the breathing period (SDP) was analyzed to determine whether temperature had any selective effects on the stability of breathing across PACAP genotypes.

Note that in adults, the pressure changes associated with the expansion of inspired air through warming and humidification dominate the pressure waveform associated with breathing. However, in small rodents, the signal is dominated by pressure changes resulting from the rarefaction and compression of gases within the airways (this point is illustrated in Fig. 3B, showing that a sizeable respiratory signal remained even when the ambient-to-body temperature differential was <1°C). While pressure changes associated with rarefaction and compression of inspired air are proportional to VT, they are also influenced by airway resistance and changes in inspiratory time. Thus, we refer to our measurements as “indices” (for detailed discussion please see Ref. 8). A further caveat of employing whole body plethysmography for this study is that the component of our signal produced by humidification and warming of inspired gas is dependent on the ambient-to-body temperature differential; reducing this differential by ambient warming is likely to have artificially decreased our VT and VE indices. Cooling the ambient environment has the opposite effect, increasing the differential (although to a much lesser extent) and having the potential to increase the magnitude of the indices. For the implications of this caveat to our main finding please see DISCUSSION.

Baseline room-air respiratory variables graphed in Fig. 2 were obtained by combining data from the 32°C baseline segment (minutes 4 and 5) of all three treatment groups (29°C, 32°C, 36°C). VT data over the entire experiment for each litter were normalized to the average baseline (minutes 4 and 5) VT index of PACAP+/− littermates (Figs. 2 and 3). In addition, to better illustrate the changes in VE after cooling, ventilatory data from minutes 19 and 20 are also expressed relative to each animal’s baseline (%change, Fig. 5).

At the end of the recording, animals were killed by decapitation, and rectal temperature (body temperature) was measured immediately thereafter using a thermocouple. Animals were then weighed and ear clipped for postanalysis genotyping, as described previously (8).

Gaseous Metabolism (V̇O₂)

To obtain the sensitivity required to record metabolic rate in freely behaving neonatal mice weighing <3 g, closed-loop, small volume, indirect calorimetry was used. The circuit consisted of a 15-ml holding container connected to an O₂ analyzer (PA-1B O₂ analyzer, Version 2; Sable Systems, Henderson, NV) calibrated to known gas mixtures. A pump (gas-analyzer, Sub-Sampler; Sable Systems), with constant flow, was used to pull air through the meter and return it to the chamber. The total volume of the circuit (~100 ml) was calculated by observing the change in % O₂ from room air, after injecting 1 ml of 100% O₂ and allowing time for the gas to equilibrate.

Experiments were performed on three sets of mice. For measurements of gaseous metabolism at 29°C (n = 39) and 32°C (n = 51), mice were separated from their mothers and preincubated in a small holding container maintained at either temperature with a heat lamp. The holding container was monitored continually with a thermocouple

![Fig. 2. Effects of PACAP genotype on baseline breathing while at thermoneutral ambient temperature (32°C). Data from minutes 4 and 5 of the baseline period, pooled from the three experimental protocols (warm, thermoneutral control, cool). Animals were breathing air and held at thermoneutral ambient temperature (32°C). A: index of tidal volume (VT). B: breathing rate (breaths/min). C: index of minute ventilation (VE). Littermate, P4 wild-type (PACAP+/−; n = 30; black bars), heterozygous (PACAP+/−; n = 70; grey bars), and knockout (PACAP−/−; n = 35; white bars) were used. In each animal, mean values were obtained from minutes 4 and 5. Error bars in this and subsequent figures are means ± SE. Note that PACAP genotype affects breathing rate (B) and overall VT index (C) but not VT index (A).](http://ajpregu.physiology.org/Downloaded from http://ajpregu.physiology.org)
(Omega Engineering), and, while in the holding container, mice were prevented from huddling. Measurements of gaseous metabolism at 36°C were made from the mice used for the 36°C plethysmography protocol (n = 50), immediately after the plethysmography protocol was completed. Therefore, animals from all three groups experienced a similar period of time at their respective temperatures prior to the determination of metabolic rate.

For all experiments, single animals were placed in the preheated metabolic chamber with the circuit open and allowed to settle for 5 minutes, after which the circuit was closed. Oxygen decay in the chamber was recorded for 5 min; however, we analyzed VO₂ only in the first minute to minimize the influence of altered inspired gases on metabolism (27, 37). Over this period, VO₂ was linear, yielding R² values >0.95. Ambient temperature within the chamber was monitored continuously with a thermocouple and maintained at 36°C, 32°C, or 29°C (±0.5°C) by making minor adjustments to the position of the heat lamp relative to the chamber. Although the metabolic rate in neonatal rats is constant over long periods of maternal separation (28), we minimized the total time of separation to a maximum of 4 h for each litter tested. Most animals appeared to become accustomed to the chamber quickly, resting without significant movement for the entire recording.

Oxygen measurements were recorded on a computer equipped with an A/D board (model TL2; Axon Instruments, Union City, CA) and data acquisition software (AxonTape, Version 2.02; Axon Instruments). At the end of the recording, animals were killed, weighed, and ear clipped for postanalysis genotyping (8).

Data Analysis

Data were analyzed in Clampfit Version 8.1. VO₂ was calculated by the following formula (derived from Fick’s principle): VO₂ = (absolute value of the difference in fractional O₂ concentration from start to end of experiment) × [(V – m) × 0.01, where V is the volume (ml) of the closed loop and m is the volume (ml) of air displaced by the animal (the volume of air displaced by an animal was assumed to be approximately equal to the animal’s mass, in grams). Values for VO₂ are normalized to the weight of the animal and expressed as ml O₂·min⁻¹·kg⁻¹, under conditions of standard temperature and pressure.

Statistical Analysis

Interactions between genotype and ambient temperature that affect breathing, VO₂, and body temperature were assessed using a two-factor, repeated-measures ANOVA, with Tukey post hoc comparisons where appropriate. Between-genotype differences in baseline breathing and animal mass were analyzed with a between-subject, one-way ANOVA. Where noted in the text, data that failed a normality test were square-root transformed before being subjected to ANOVA.

RESULTS

Three 20-min plethysmography protocols were used in which ambient temperature was: 1) increased from 32°C to 36°C (warm), 2) held constant at 32°C (thermoneutral control), or 3) decreased from 32°C to 29°C (cool) (Fig. 1A). Neonates used for the warm plethysmography protocol were subsequently subjected to the metabolism protocol at an ambient temperature of 36°C. Two separate sets of animals were used for measuring VO₂ at 32°C and 29°C. The total ratio of PACAP⁺/⁺, ⁺/⁻, and ⁻/⁻ genotypes for all experiments was 48:122:55, respectively, confirming our previous observation that there was no increase in the mortality for PACAP-deficient mice at P4 (8). The mass of PACAP⁻/⁻ neonates was ~88% that of PACAP⁺/⁺ and PACAP⁺/⁻ littermates (P < 0.001; Fig. 1C). However, there was no difference in body mass between PACAP⁺/⁺ and PACAP⁺/⁻ animals (P = 0.61).

Effects of PACAP Deficiency on Breathing During Thermoneutral Conditions

Minutes 4 and 5 of each of the three plethysmography protocols (when all animals experienced identical conditions) were combined to provide the maximum power to examine initial baseline breathing at thermoneutral temperature (32°C, Fig. 2, A–C). There were no significant differences in VT index between genotypes (Fig. 2A; P = 0.153). However, both PACAP⁺/⁻ and PACAP⁻/⁻ mice breathed at a slightly lower rate (Fig. 2B; P = 0.018), reducing overall baseline VE significantly compared with wild-type littermates (Fig. 2C; P = 0.044, data square root transformed). PACAP deficiency had no effect on SDP during baseline (P = 0.172, not shown).

To assess the changes in breathing resulting from the time in the chamber (time control), 36 animals were exposed for 20 min to a constant ambient temperature of 32°C. Similar to the genotypic differences seen in minutes 4 and 5, both PACAP⁺/⁻ and PACAP⁻/⁻ neonates continued to have a reduced VE index throughout the time control (P = 0.023, Fig. 3A, right). Although a reduced rate may have contributed to this effect, the effect of genotype on rate alone was not quite significant (P = 0.08, Fig. 3A, middle). Also mirroring minutes 4 and 5, over the entire 20-min control period genotype alone had no significant effects on VT index (Fig. 3A, left) or breathing stability (not shown).

While there were no significant effects of time alone on rate or overall VE index (rate: P = 0.077, Fig. 3A, middle; index of VE: P = 0.09, Fig. 3A, right), there was a significant interaction between genotype and time on VT index (P = 0.021): the wild-type VT index was significantly reduced at minutes 19 and 20, relative to baseline (minutes 4 and 5) (Fig. 3A, left, filled bars; −10.1% ± 5.7%; Tukey post hoc: P = 0.012); however, there was no effect of time on the VT index of PACAP-deficient animals (Fig. 3A, left; PACAP⁺/⁺: +5.7% ± 3.2%; Tukey post hoc: P = 0.324; PACAP⁻/⁻: +1.6% ± 7.5%; Tukey post hoc: P = 0.566).

In summary, PACAP-deficient animals have reduced VE at thermoneutral ambient temperatures, but any changes with time in rate, VT index, and VE index were slight at best and below significance. The only significant effect of time was in wild-type animals; despite being kept at their thermoneutral ambient temperature, VT index dropped by 10%.

Effects of PACAP Deficiency on Breathing After a Mild Increase in Ambient Temperature

Increasing ambient temperature from 32°C to 36°C significantly affected all breathing parameters (VT index, rate, and VE index decreased: P < 0.001 for each variable; Fig. 3B; SDP increased: P < 0.001, not shown). However, PACAP genotype had no significant effect on the magnitude of these responses (genotype × temperature interaction; P > 0.05 for all variables).

Effects of PACAP Deficiency on Breathing After a Mild Reduction in Ambient Temperature

When considering all animals together, lowering ambient temperature had a significant overall effect on VT index (P =
Fig. 3. Breathing of PACAP genotypes over time and after warming and cooling of the ambient temperature. Breathing during 20 min at thermoneutral (32°C) ambient temperature (thermoneutral time control, n = 36; A), during and after warming the ambient temperature to 36°C (warming, n = 45; B), and during and after cooling ambient temperature to 29°C (cooling, n = 54; C). Data shown are for index of VT, (left), breathing rate (Breaths/min, middle), and index of minute V̇E (right) during minutes 4 and 5 (baseline), minutes 7 and 8 (temperature cooling or warming), and minutes 19 and 20 (new steady-state temperature) from wild-type (PACAP+/+; black bars), PACAP heterozygous (PACAP+/−; grey bars), and PACAP knockout (PACAP−/−; white bars) P4 littermates. Significant effects (P < 0.05) of time, genotype (G), and genotype-time interaction (G × time) were determined using two-factor, repeated-measures ANOVAs, and are indicated in each graph. Note that 1) in PACAP-deficient animals, neither the index of VT nor the index of V̇E changes with time (A); 2) the absence of any genotype-specific effects of warming on breathing (B), and that cooling decreases the indices of VT and V̇E in PACAP-deficient animals, but not in wild-type littermates (C).

0.003, Fig. 3C, left), rate (P < 0.001, Fig. 3C, middle), and V̇E index (P < 0.001, Fig. 3C, right). Cooling also significantly reduced breathing stability (i.e., SDP increased, P = 0.016, not shown). The effects of temperature on rate and variability were independent of genotype (genotype × temperature interaction on rate and SDP: P = 0.730 and P = 0.08, respectively). Importantly, however, the responses of VT and V̇E indices were dependent on genotype. In wild-type animals, cooling had no significant effect on either VT or V̇E indices, whereas in their PACAP-deficient littermates, cooling caused a reduction in
Effects of Initial VT on the Response of PACAP-Deficient Animals to Cooling

At the start of the experiment (minutes 4 and 5), when all three groups experience the same conditions, PACAP-deficient animals in the thermoneutral control group had a slightly reduced breathing rate compared with that of the PACAP-deficient animals in the cooling group (difference: 10% and 16% for PACAP+/+ and −/− animals, respectively; compare Fig. 3A, middle with Fig. 3C, middle). We explored the effect of this slight difference in initial phenotype to determine whether the reduced VE experienced by PACAP-deficient animals with cooling was the result of an initial high level of VE. Specifically, we analyzed the relationship between initial ventilatory frequency and change in VE over time alone (control group, Fig. 7A) and during cooling (cooling group, Fig. 7B). In the control group, the fall in VE over time was only weakly related to the initial level of VE, and this effect was confined to PACAP+/+ animals (Fig. 7A; PACAP+/+: $R^2 = 0.48$, $P < 0.01$). Moreover, in the cooling group, the reduction in VE observed in PACAP-deficient animals was unrelated to the initial rate (Fig. 7B; PACAP+/+; $R^2 = 0.04$, $P = 0.28$; PACAP−/−; $R^2 < 0.001$; $P = 0.98$).

DISCUSSION

In mice, PACAP deficiency increases the chance of sudden neonatal mortality in the second postnatal week, especially when litters are exposed to mild thermal stress (12). Our previous study demonstrated that during anesthetic-induced hypothermia, PD7–14 PACAP-null mice, but not their wild-type littermates, experience long-duration apneas, ultimately leading to atrioventricular block (8). However, breathing, metabolic rate, and body temperature are tightly coupled, and PACAP has been implicated in the regulation of all three physiological systems (1, 8, 12). In the present study, we investigated the relative effect of PACAP-deficiency on the adaptation of these systems to thermal stress free from the confounding effects of anesthetic and at P4, a developmental time point before the increased mortality for PACAP−/− mice.

Effects of PACAP Deficiency on Gaseous Metabolism and Body Temperature

Changing ambient temperature in either direction significantly increased VO$_2$ in all genotypes ($P < 0.001$), suggesting that 32°C was within the thermoneutral range for these animals (Fig. 6A). However, all genotypes had the same metabolic response to temperature changes (genotype × temperature interaction: $P = 0.501$, Fig. 6A).

At ambient temperatures of 29°C, 32°C, and 36°C, the body temperatures of neonates were ~32–33°C, 35–36°C, and 37°C, respectively. There was neither an effect of genotype alone ($P = 0.580$) nor an interaction between genotype and ambient temperature on body temperature ($P = 0.321$, Fig. 6B).

Figure 4. Representative plethysmographic tracings, showing the breathing pattern of wild-type and PACAP+/− littermates before, during, and after ambient cooling. Recordings of entire experiments are shown (top), including the 5 min of baseline breathing ($T_a = 32°C$), the period of cooling (between dotted lines), and breathing at the reduced temperature ($T_a = 29°C$). Expanded traces of individual breaths from minutes 4 and 5 ($T_a = 32°C$) and minutes 19 and 20 ($T_a = 29°C$) are indicated below, illustrating the reduction in VT experienced only by PACAP-deficient animals with cooling, while the VT of the wild-type littermate is maintained. The VT response of PACAP−/− littermates is similar to that of PACAP+/− animals (not shown).
This study presents two novel findings. First, unlike their wild-type littermates, the VT and V\(\dot{E}\) of P4 mice with both complete and partial PACAP deficiency are significantly reduced during cooling. Second, PACAP deficiency has no effect on the metabolic and body temperature responses of P4 neonates to changing ambient temperature. Thus, while the inability to measure blood gases means we cannot conclude unequivocally that PACAP deficiency results in hypventilation during cooling, PACAP-deficient mice show a relative hypventilation, suggesting that they are more prone to hypoxemia and hypercapnia during cooling compared with wild-type littermates.

**Methodological Considerations**

There are several caveats that should be considered when interpreting our data.

**Maternal separation.** For both plethysmography and indirect calorimetry experiments, some animals were separated from maternal care for several hours. The possibility exists, therefore, that the metabolism and/or breathing of some pups was compromised prior to testing. To ensure there was no experimenter-introduced bias with regard to the average duration of maternal separation for each genotype, we tested pups randomly while blinded to genotype. To determine whether the PACAP genotype affected how animals respond to maternal separation, we performed time-control experiments in which animals were maintained at thermoneutral conditions. The results indicate that V\(\dot{E}\) was relatively constant when animals were maintained at thermoneutral ambient temperature, regardless of genotype (Fig. 5A). Thus, PACAP genotypic differences in anxiety levels (33) or other physiological manifestations resulting from maternal separation cannot alone explain the precipitous fall in V\(\dot{E}\) observed in the cool-challenged, PACAP-deficient animals.

**Comparison with previous study.** Although PACAP-deficient animals in both this study and a previous study (8) had lower V\(\dot{E}\) than their wild-type littermates during baseline conditions, in the present study, this was due to a reduction in VT, not V\(\dot{E}\) as was found previously. We currently do not know enough about the specific role of PACAP in the control of breathing to explain this difference with any degree of satisfaction. However, the effect is probably not due to a difference in settling time since, in the present study, the VT of each genotype converged over the course of the 20-min thermoneutral protocol (Fig. 3A). We note that strain can have a considerable effect on breathing in neonatal rodents (5, 10) and speculate that the proportion of 129SvJ alleles to C57/BL6 alleles at loci important for ventilatory control may have changed between studies.

**Effect of differences in body weight.** The P4 PACAP\(^{-/-}\) neonates used in the present study were significantly smaller than their wild-type and heterozygous littermates, a phenotypic difference not observed in other studies using PACAP signaling-deficient mice (8, 11, 32). Therefore, the possibility exists that the reduction in VT and V\(\dot{E}\) in PACAP\(^{-/-}\) neonates with cooling is related to a smaller body size or is secondary to a developmental defect. However, we feel this is unlikely because PACAP\(^{+/-}\) neonates are the same size as wild-type littermates but share the ventilatory phenotype of their PACAP\(^{-/-}\) littermates.

**Technical considerations in assessing respiratory measurements.** As stated in METHODS, the pressure signal recorded from small animals in a chamber is likely dominated by rarefaction and compression of gases in the lungs. However, if the signal includes a component derived from the heating and humidification of inspired air, then changing the ambient temperature could have directly influenced the size of the recorded signal. Indeed, we note that with warming, which reduces the ambient-
to-body temperature differential, both VT and V̇E indices fell, despite the fact that metabolism, and therefore V̇E, should increase with temperature. However, regarding our main finding that PACAP plays a role in maintaining V̇E during cooling, we note 1) that the effect is genotype-specific [Given that PACAP-genotype has no effect on body temperature (see Fig. 6), the effects of cooling on the proportion of the signal sensitive to the ambient-to-body temperature differential would be expected to affect all genotypes equally.]; and 2) that falls in ambient temperature exacerbate the disparity between ambient and body temperature, which would be expected to increase the component of the signal originating from the heating of inspired air; all else being equal, the signal should increase. However, the signal recorded for PACAP-deficient animals decreased as temperature fell.

A related consideration is that the amplitude of the rarefaction- and compression-derived pressure signal is, in part, subject to airway resistance; the higher the resistance, the greater the amplitude of the signal. PACAP is a bronchodilator (23) and, thus, PACAP-deficient mice may be more prone to bronchoconstriction than wild-type littermates and have a higher airway resistance. Yet, PACAP-deficient mice had a smaller VT index than wild-type littermates. This argument suggests that our results might underestimate the reduction in V̇E caused by cooling in PACAP-deficient animals.

**Response of V̇O₂ and Body Temperature to Changes in Ambient Temperature and the Role of PACAP**

The body temperature measurements made in the present study demonstrate that, despite the augmentation of metabo-

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**Fig. 6.** Effect of mild changes in ambient temperature on the metabolic rate and body temperature (T_B) of PACAP genotypes. Oxygen consumption (V̇O₂) (A), and body temperature (T_B), at ambient temperatures of 29°C, 32°C, and 36°C in P4 PACAP genotypes. PACAP+/−: n = 8 (29°C); n = 10 (32°C); n = 14 (36°C); black bars. PACAP+−−: n = 19 (29°C); n = 33 (32°C); n = 22 (36°C); grey bars. PACAP−−−: n = 12 (29°C); n = 8 (32°C); n = 14 (36°C); white bars. Significant effects (P < 0.05) of ambient temperature (T), genotype, and genotype-ambient temperature interactions were determined with a 2-factor, repeated-measures ANOVA, and are indicated in each graph. Note the lack of significant differences between PACAP genotypes with respect to oxygen consumption and body temperature.

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**Fig. 7.** Influence of initial breathing frequency on the ventilatory response of PACAP genotypes. Relationship between initial ventilatory rate during the baseline period (minutes 4 and 5 of protocol, T_a = 32°C) and the change in V̇E (% change from baseline to minutes 19 and 20 of the protocol) during the thermoneutral time control (A) and after cooling (B) in PACAP+/− (solid circles), PACAP−− (open circles) animals, and wild-type littermates (triangles). During cooling, linear regression indicates no significant relationship for either PACAP+/− animals (R² = 0.04, P = 0.28), PACAP−− animals (R²<0.01, P = 0.99), or wild-type littermates (R² = 0.18, P = 0.20). Thus, the reduction in V̇E experienced by PACAP-deficient genotypes with cooling is unrelated to an initially high level of V̇E.
lism at reduced ambient temperature, neonatal mice depend heavily on behavioral mechanisms for maintaining body temperature. Thus, they confirm studies suggesting neonatal rodents are poikilothermic (28).

The values we obtained for thermonutral $V_02$ are within the range previously reported for neonatal rodent of equivalent age (3, 29), as are the changes observed in gaseous metabolism after warming and cooling (28). The increase in $V_02$ at an ambient temperature of $29^\circ C$ is likely a result of adaptive thermogenesis, while the increase at $36^\circ C$ is likely a $Q_{10}$ effect. The lack of differences between genotypes for both $V_02$ and body temperature imply that PACAP has no effect on adaptive thermogenesis at this developmental stage.

Increased Mortality of PACAP$^{-/-}$ Neonates with Lower Ambient Temperature

Several recent studies have noted that absence of PACAP and/or PAC1 receptors leads to a higher mortality prior to weaning (11, 13, 14, 17), a condition exacerbated by raising the animals in rooms kept at slightly lower temperatures (12). In this paper, we show that nonanesthetized PACAP$^{-/-}$ and PACAP$^{+/+}$ animals express a temperature-dependent respiratory phenotype by P4. However, while both PACAP$^{-/-}$ and PACAP$^{+/+}$ animals share the abnormal temperature-dependent respiratory phenotype, only PACAP$^{-/-}$ neonates are reported to have significant mortality with mild reductions in ambient temperature. Thus, it is likely that additional defects associated with complete PACAP deficiency contribute to death. These include a more pronounced blunting of the hypoxic and hypercapnic ventilatory responses at P4 (8) and abnormalities in temperature regulation, fat metabolism, and cardiac function that occur later on in postnatal development (11–13, 32, 39). Interestingly, some of the characteristics of PACAP$^{-/-}$ and PAC1 receptor$^{-/-}$ neonates that appear later on in development (11, 32) resemble those of neonatal rats exposed to chronic hypoxia from birth. These similarities include growth inhibition, pulmonary hypertension, and neonatal death starting at P5 (see Figs. 5.25 and 5.26 in Ref. 27). Therefore, the phenotypes appearing after P4 in PACAP$^{-/-}$ neonates may be secondary to chronic hypoxemia from birth, related to insufficient ventilatory responses to environmental stress.

Mechanism of Action of PACAP in Breathing During Cooling

When P4 neonatal mice are exposed to a cool ambient environment, body temperature falls, despite increased thermogenesis. Thus, a complement of factors that counteract the inhibitory effects of $Q_{10}$ on $V_02$ may be important. Although PACAP appears to be one of these critical factors, the cellular loci where PACAP is acting remains speculative. One possibility is that the impaired chemoreflex in PACAP-deficient mice that we previously reported (8) is exacerbated by cooling. Several reports suggest that intravenous injections of PACAP can have acute effects on $V_02$ in adult animals, possibly by acting directly on the carotid body (15, 35). Interestingly, the carotid bodies have also been implicated in thermoregulation (20). It is equally possible that PACAP maintains $V_02$ by stimulating neurons that are activated by reductions in ambient and/or body temperature but do not participate directly in the chemoreflexes. We note that neurons within the preoptic and paraventricular nuclei of the hypothalamus that are involved in temperature regulation also project to and stimulate phrenic motoneurons (6, 19, 24, 25) and receive dense innervation from PACAP-positive axons (2, 9, 34). Finally, it is also possible that the effect of PACAP on the ventilatory responses to cooling are indirect, being mediated by other neuropeptides or hormones. Numerous studies across multiple species indicate that PACAP is produced in the adrenomedulla and can stimulate both the production and release of catecholamines (7, 16, 18, 21, 26, 40, 44). Further, PACAP appears especially efficacious in evoking release of catecholamines during the sympathetic response to several forms of physiological stress (13, 22, 45).

Perspectives and Significance

In this study, we have demonstrated an important role for PACAP signaling in the preservation of neonatal $V_02$ during mild temperature stress. These observations may partially explain the temperature-dependent mortality of PACAP mice, and are in keeping with a growing consensus that PACAP is important for maintaining homeostasis in the face of acute environmental stress: a ubiquitous requirement of all animals that might contribute to its highly constrained structure throughout evolution.

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