A Whole-Genome Scan for 24-Hour Respiration Rate: A Major Locus at 10q26 Influences Respiration During Sleep

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Identification of genes causing variation in daytime and nighttime respiration rates could advance our understanding of the basic molecular processes of human respiratory rhythmogenesis. This could also serve an important clinical purpose, because dysfunction of such processes has been identified as critically important in sleep disorders. We performed a sib-pair–based linkage analysis on ambulatory respiration rate, using the data from 270 sibling pairs who were genotyped at 374 markers on the autosomes, with an average distance of 9.65 cM. Uni- and multivariate variance-components–based multipoint linkage analyses were performed for respiration rate during three daytime periods (morning, afternoon, and evening) and during nighttime sleep. Evidence of linkage was found at chromosomal locations 3q27, 7p22, 10q26, and 22q12. The strongest evidence of linkage was found for respiration rate during sleep, with LOD scores of 2.36 at 3q27, 3.86 at 10q26, and 1.59 at 22q12. In a simultaneous analysis of these three loci, >50% of the variance in sleep respiration rate could be attributed to a quantitative-trait loci near marker *D10S1248* at 10q. Genes in this area (*GFRA1, ADORA2L, FGR2, EMX2,* and *HMX2*) can be considered promising positional candidates for genetic association studies of respiratory control during sleep.

Introduction

Billions of mammals, including humans, depend on rhythmic breathing to regulate one of the foremost aspects of homeostasis: the appropriate exchange of oxygen and carbon dioxide. Although a number of theoretical models have been proposed, the actual mechanisms responsible for respiratory rhythmogenesis were largely misunderstood until recently, when important new insights were gained from in vitro studies of brain stem preparations from neonatal rodents. In the rostral ventrolateral medulla, a set of neurons known as the "preBötzinger complex" act as an inspiratory pacemaker that plays a vital role in respiratory rhythmogenesis (Rekling and Feldman 1998; Richter and Spyer 2001; Feldman et al. 2003). Bilateral outflow of the preBötzinger complex and its associated "distributed" network in the lower brain stem is transmitted to the spinal motor neurons (hypoglossal nerve and phrenic nerve) to produce rhythmic contraction.

Although in vitro models, with their elimination of the many complex peripheral inputs, allow reliable modeling of the neuronal pacemaker network in the preBötzinger complex, it has been widely recognized that they yield an oversimplified picture of the true in vivo generation of the respiratory rhythm (Richter and Spyer 2001; Hilaire and Pásaro 2003). An alternative way to access the molecular biology of respiration is to characterize the genetic variation involved in individual differences in the control of respiratory behavior. This genetic variation should generate clues to the signaling pathways in respiratory neurons by providing molecular targets for animal-genetics engineers to produce conditional knockouts or transgenic animals that would allow detailed anatomical tracing of the neural network involved in respiratory control. To ensure that the investigated pathways have a meaningful concomitant in humans, it would be advantageous to identify genes for respiration in a human population, preferably through measuring respiration under natural conditions.

Understanding the basic molecular processes in human respiratory rhythmogenesis serves an important clinical purpose, because such processes have been identified as critically important in sleep apnea (Hanly 1992; American Academy of Sleep Medicine Task Force 1999; Palmer and Redline 2003). Sleep apnea is defined as repetitive episodes of decreased or total cessation of respiratory airflow during sleep, leading to a >4% fall

Table 1

Ambulatory Respiration Rates in Male and Female Siblings during Three Daytime Periods and Nighttime Sleep

SUBJECT GROUP AND STATISTIC	AGE (years)	RESPIRATION RATE (breaths/min) DURING			
		Morning	Afternoon	Evening	Night
Female:					
Mean	32.14	16.40	16.79	17.36	15.98
SD	11.42	1.27	1.31	1.53	2.04
n	186	179	182	182	176
Male:					
Mean	32.09	16.11	16.66	17.37	15.10
SD	10.79	1.21	1.29	1.61	1.97
n	120	118	117	118	111
All:					
Mean	32.12	16.29	16.74	17.36	15.64
SD	11.16	1.25	1.30	1.56	2.06
n	306	297	299	300	287

NOTE.—To remove the effects of differences in physical-activity pattern during the awake periods, respiration rates were computed using data obtained only when subjects were sitting.

in oxygen saturation and to sleep fragmentation. Sleep apnea can be central or obstructive. Obstructive sleep apnea is caused by upper-airway collapse during inspiration and is accompanied by strenuous breathing efforts. In central sleep apnea, there are unknown primary defects in the central respiratory rhythmogenesis and/or the chemosensitive control mechanisms that lead to diminution or cessation of thorac-abdominal respiratory movements. Sleep apnea constitutes a major public health problem because of its high prevalence and its association with cardiovascular morbidity (Wolk et al. 2003).

Recently, we completed a large twin-sibling study to test the heritability of 24-h respiration rate (H. M. Kupper, G. Willemsen, D. Posthuma, D. De Boer, D. I. Boomsma, E. J. C. de Geus, unpublished data). We used ambulatory recording of the thorax impedance to obtain the respiration signal. This allows measurements in natural settings and, importantly, is sufficiently unobtrusive to allow recording during sleep (de Geus et al. 1995; de Geus and Van Doornen 1996). Heritability of respiration rate during the daytime was moderate (41%–50%), whereas heritability at night was high (81%).

Here, we report a whole-genome scan on ambulatory respiration rate, using the data from 270 sibling pairs who were genotyped at 374 marker loci on the autosomes, with an average distance of 9.65 cM (Kosambi). Variance-components–based, multipoint, model-free linkage analyses were performed separately on respiration-rate data obtained during three daytime periods (morning, afternoon, and evening) and during nighttime sleep.

Subjects and Methods

Subjects

In 1991, the Netherlands Twin Register started a longitudinal survey study of health and lifestyle (Boomsma et al. 2002). Questionnaires were sent out in 1991, 1993, 1995, and 1997 to adolescent and adult twins and their family members. Twin pairs were asked to participate in all years; parents were asked to participate in 1991, 1993, and 1995; and siblings were included in 1995 and 1997. On the basis of questionnaire data on anxiety and depression, a genetic-factor score was composed that was used for extreme discordant and concordant selection for a QTL study of anxious depression (Netherlands Twin Family Study of Anxious Depression; see Boomsma et al. [2000] for a detailed description). Primary ascertainment of families was through these extremely discordant or concordant sib pairs, but all other siblings in the family were invited to participate. The distribution of anxious depression in the resulting sample ($N = 2,724$), therefore, was near normal with only mild kurtosis, in comparison with that of the entire unselected sample (see fig. 2 in Boomsma et al. 2000).

All subjects and their parents were asked to provide a buccal swab for DNA isolation. Of the 1,962 subjects (72%) who returned a buccal swab, 917 (624 offspring and 293 parents) were genotyped at 379 markers on the autosomes. Subjects for whom <50% of the markers were successfully typed were removed from the sample. This resulted in a subsample of 558 offspring and 278 parents from 192 families for whom genotyping was successful. From the offspring who were successfully genotyped, 306 subjects from 123 families participated in 24-h ambulatory monitoring. For a total of 270 complete sib pairs, ambulatory respiratory signals and adequate marker data were available. Sib pairs could be full DZ twin pairs, an MZ or a DZ twin paired with a singleton sibling, or two singleton siblings. From MZ pairs, data from only one (randomly chosen) twin was used for genotyping and the linkage analyses.

Subjects gave written informed consent, and both the DNA sampling and the ambulatory protocol were approved by the institutional review board of the Vrije Universiteit Medical Center.

Ambulatory Recording

Ambulatory recording of the thorax impedance (Z) was performed by the Vrije Universiteit Ambulatory Monitoring System (VU-AMS) with a six-spot electrode configuration (de Geus et al. 1995; de Geus and Van Doornen 1996). Two electrodes on the back were used to continuously send a high frequency current of 50 kHz 350 μA through the subject. Two electrodes on the chest
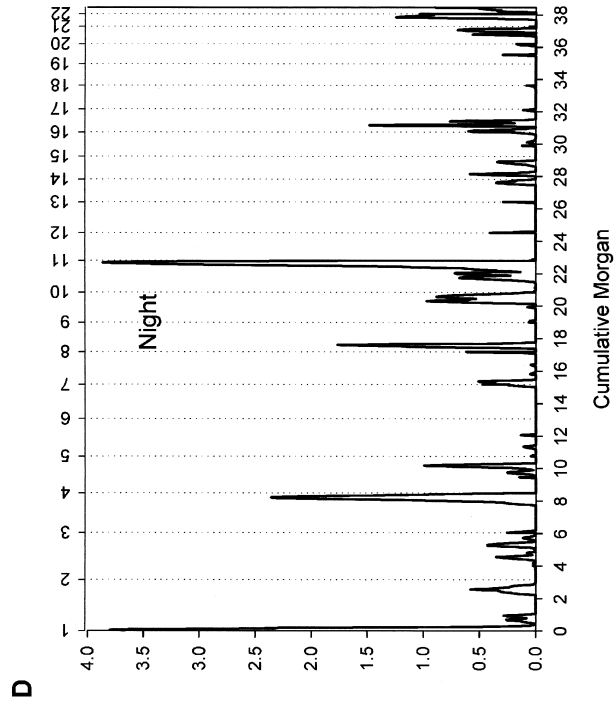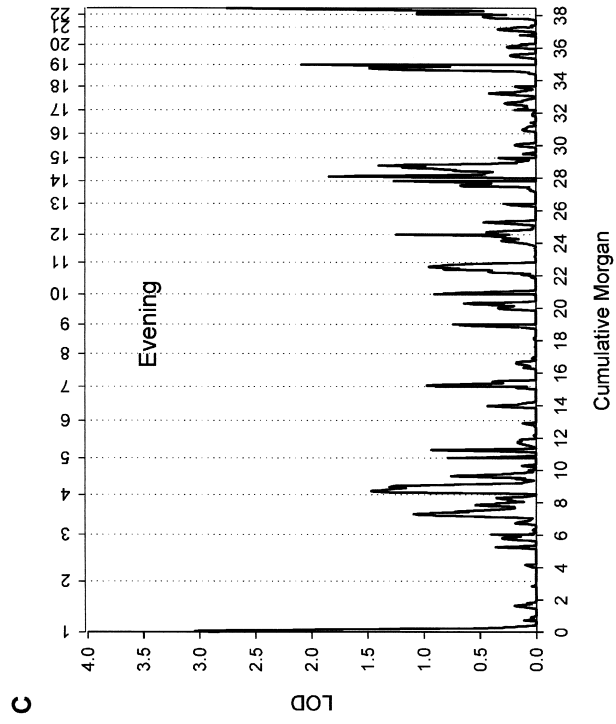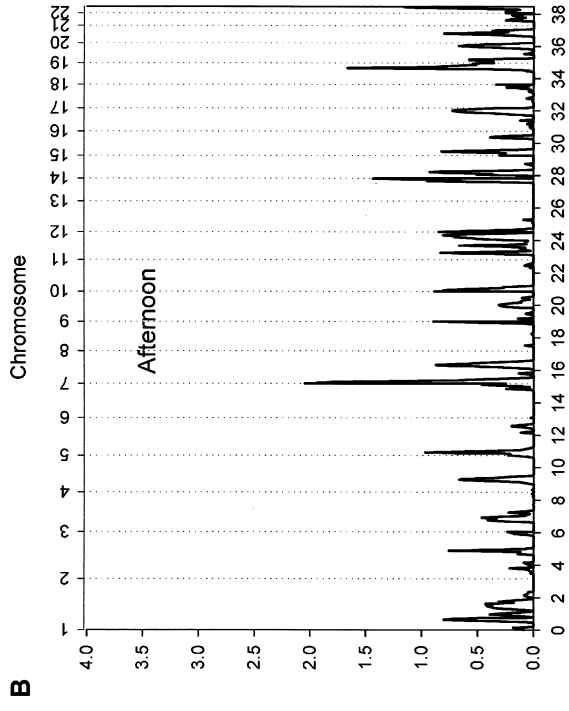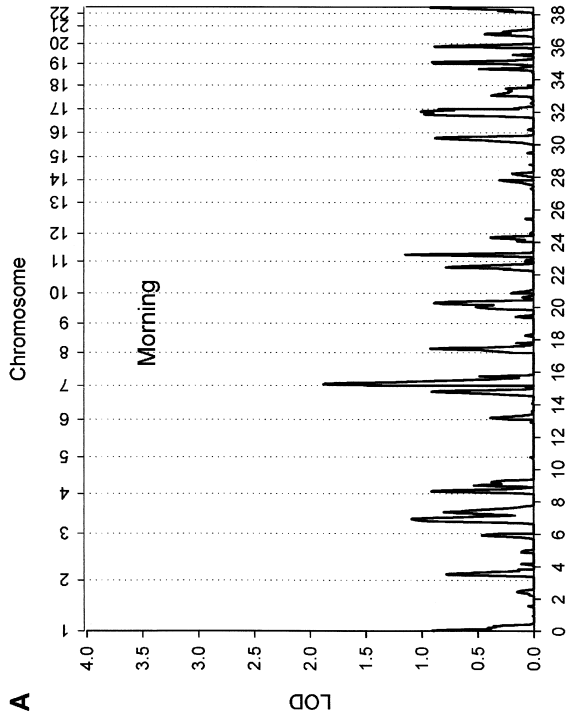
were used to measure impedance. The upper measuring electrode was placed at the jugular notch of the sternum, between the collarbones. The lower measuring electrode was placed at the tip of the sternum (xiphoid process). The upper current electrode on the back was placed at least 3 cm *above* the horizontal plane of the upper measuring electrode. The lower current electrode on the back was placed at least 3 cm *below* the horizontal plane of the lower measuring electrode. The thoracic impedance signal was amplified and led to a precision rectifier. The rectified signal was filtered at 72 Hz (low pass) to give basal thoracic impedance, Z_0. Filtering of Z_0 at 0.1 Hz (high pass) supplies the dZ signal, which contains three major components: the high frequent impedance changes due to the ejection of blood into the aorta during systole; the low frequent impedance changes due to arm and upper-body movement; and, in between these frequencies, the thoracic impedance changes due to respiration. Fragments of 100-s dZ signal were band-pass filtered with 0.1 and 0.4 Hz cutoffs after being tapered with $[\sin(x)]^2$, yielding the respiration signal. VU-AMS software automatically detects the start of inspiration and expiration for each breath and displays the respiration signal with these markers for interactive visual inspection.

During the subject's awake time, the VU-AMS produces an audible alarm approximately every 30 min (± 10 min randomized), to prompt the subject to fill out an activity diary. Subjects were instructed to write down the time and description of their activities and bodily postures during the past 30-min period, in chronological order. Information from the diary about physical activity and posture was combined with the body-movement signal from a built-in vertical accelerometer, to specify accurately the start and end times of any activity/posture changes of the subjects. We divided the entire recording into smaller fragments that were completely stationary with regard to physical activity and posture—for example, within each fragment, no shifts in posture occurred. Each fragment was coded for posture (lying, sitting, standing, walking, or bicycling), activity (e.g., deskwork, housekeeping, or watching TV), and location (e.g., at home, at work, or at a public place). The coded fragments were never <5 min or >1 h. On the basis of the reported times of awakening, lunch, dinner, and bedtime, data were aggregated across four periods: morning, afternoon, evening, and nighttime sleep. In 8% of the subjects, the exact time of awakening, lunch, dinner, or bedtime could not be extracted from either the diary or

body movement. For these subjects, the missing time was imputed by use of the mean times of these events in the rest of the sample.

For the nighttime recording, the average respiration rate was aggregated across all valid breaths. For the three active periods of the day, the average respiration rates were computed using only the valid breaths obtained when subjects had been sitting. The use of sitting-only data eliminates the influence of individual differences in daytime physical-activity patterns, which are known to have a very strong effect on respiratory behavior. Total duration of sitting activities averaged 115 min in the morning, 152 min in the afternoon, and 137 min in the evening. In 34 subjects, one or more periods of the day were missing for the final analyses. This was because of equipment failure in a few cases, but it was mostly because the impedance signal quality was visually judged as insufficient for adequate respiration scoring. Consequently, the number of subjects varies slightly across the four periods (see table 1).

DNA Genotyping and Error Checking

Genotyping was conducted by the Marshfield Laboratory using the 10-cM spaced microsatellite screening set 10 (Yuan et al. 1997), with few alternative markers. Pedigrees were checked for Mendelian errors by use of the program Unknown (Schaffer 1996), and pedigree relationships in the entire sample were checked by use of the program GRR (Abecasis et al. 2001). Mendelian errors were removed by assigning missing values to the marker genotypes if the errors appeared incidental. Recombination likelihoods were checked using the Merlin program (Abecasis et al. 2002). Excessive recombinations were observed for 5 of the 379 autosomal markers that indicated potential problems: two markers on chromosome 1 (*D1S160* and *D1S1627-ATA25E0*), two markers (*D11S1985-GGAA5C04* and *D11S2006-GATA46A12*) in a group of five very closely or identically mapped markers on chromosome 11, and one marker on chromosome 20 (*D20S159-UT1307*). These five markers were excluded, leaving a final set of 374 markers for the analyses. For all other recombination problems, the data were cleaned using the default procedure of the Merlin program.

Marker distances in cM (Kosambi) were assigned by use of the deCODE map (Kong et al. 2002), when available. For markers not mapped by deCODE, the original

Figure 1 Univariate, multipoint, variance-components linkage of the 22 autosomes in 270 sib pairs. Linkage results are presented separately for the morning *(A)*, afternoon *(B)*, evening *(C)*, and nighttime *(D)* and are adjusted for mean effects of sex and age on respiration rate. The X-axis plots genetic distance in cM (Haldane), and the Y-axis represents the LOD score.

Table 2

Main Linkage Findings for Ambulatory Respiration Rate

	LOCATION	LOD Score (P)[a] for Period				
Chromosome	(cM)	Morning	Afternoon	Evening	Night	Surrounding Markers
3	217	.00	.00	.26	2.36 (.080)	D3S1262, D3S2398
7	4	1.70 (.083)	1.94 (.023)	.94	.40	D7S2477, D7S3056
10	182	.04	.00	.74	3.86 (.004)	D10S1222, D10S1248
22	39	.76	.89	2.75 (.001)	.87	D22S689, D22S683

[a] For LOD scores >1.5, the empirical chromosome-wide *P* values are given (in parentheses) as obtained from 1,000 permutations of the data set.

distance provided on the Marshfield Web site (Broman et al. 1998) was transformed by linear interpolation from adjacent markers with known deCODE map values.

Linkage Analysis

Variation in respiration rate was decomposed into variation due to QTLs (σ_q^2), variation due to additive influences (σ_a^2), and variation due to nonshared environmental influences (σ_e^2), by use of structural equation modeling as implemented in the Mx software package (see Mx software Web site). On the basis of genetic model fit in the full MZ and DZ twin samples, a model with additive genetic influences and unique environmental influences was known to best describe the twin covariance structure (H. M. Kupper, G. Willemsen, D. Posthuma, D. De Boer, D. I. Boomsma, E. J. C. de Geus, unpublished data). Age and sex were included as covariates in the model, to reduce residual variation in respiration rate and to increase power to detect linkage to a QTL. The formal model is represented by RR = $\mu + \beta_1 \times$ sex $+ \beta_2 \times$ age $+ \varepsilon$, where RR is the observed respiration rate, μ is the intercept or grand mean, β_1 is the deviation of males from females, β_2 is the regression weight of age on respiration rate, and ε is the residual variance not explained by age or sex. The residual variance is divided into variance due to additive polygenetic variance; environmental variance, including measurement error; and variance associated with a putative QTL. Estimates of the variance component associated with a putative QTL were obtained by using the $\hat{\pi}$ approach, in which the covariance due to the marker or trait locus for a sib pair is modeled as a function of the estimated proportion of alleles shared identical by descent (IBD). The general variance-covariance matrix for pair *j,k* of the *i*th family (Ω_{ijk}) is then given by

$$\Omega_{ijk} = \begin{cases} \sigma_a^2 + \sigma_q^2 + \sigma_e^2 & \text{if } j = k \\ \rho\sigma_a^2 + \hat{\pi}_{ijk}\sigma_q^2 & \text{if } j \neq k \end{cases},$$

where ρ denotes the expected overall IBD proportion. The probabilities of sharing 0, 1, or 2 alleles IBD at

every 1 cM (Haldane) over the genome were estimated by use of Merlin (Abecasis et al. 2002).

The effect of the QTLs was evaluated by the LOD score, computed as $-2\ln$ LR/4.6, where LR is the likelihood ratio. Descriptive significance levels for $-2\ln$ LR are obtainable from its asymptotic distribution, which is a 50:50 mixture of χ^2 random variables with 1 and 0 df (Self and Liang 1987; Sham 1998, p. 265). For chromosomes with promising findings, however, empirical *P* values were determined by simulation, using the 1000 × permutation algorithm as described by Lystig (2003).

In addition to the variance-components analyses discussed above, we reran the univariate linkage scans in Merlin Regress (Sham et al. 2002). Since virtually identical results were obtained from both procedures, only the Mx analyses will be presented here, for the sake of brevity. The Mx variance-components approach was chosen because it allowed additional multivariate and multilocus analyses.

Results

Table 1 shows the statistics of the genotyped sample of male and female offspring. Equating the means for males and females yielded a model with a significant loss of fit ($\chi_4^2 = 20.76$; $P < .001$). The main effect of sex shows a slightly slower respiration in males than in females. Respiration rate in all periods decreased with age, but the correlation was significant only for males in the morning sample ($r = -0.20$). Correlations between respiration rate and a summary factor score for anxious depression were nonsignificant (r values between -0.02 and 0.03). Heritabilities based on the sibling correlations were 29% in the morning, 37% in the afternoon, 43% in the evening, and 81% at night. Respiration rates from the four periods were significantly correlated (all values of *r* between 0.27 and 0.65).

Genomewide Linkage Analysis

Figure 1 shows the results of the autosomal whole-genome scan. The highest peak (LOD score = 3.86) was
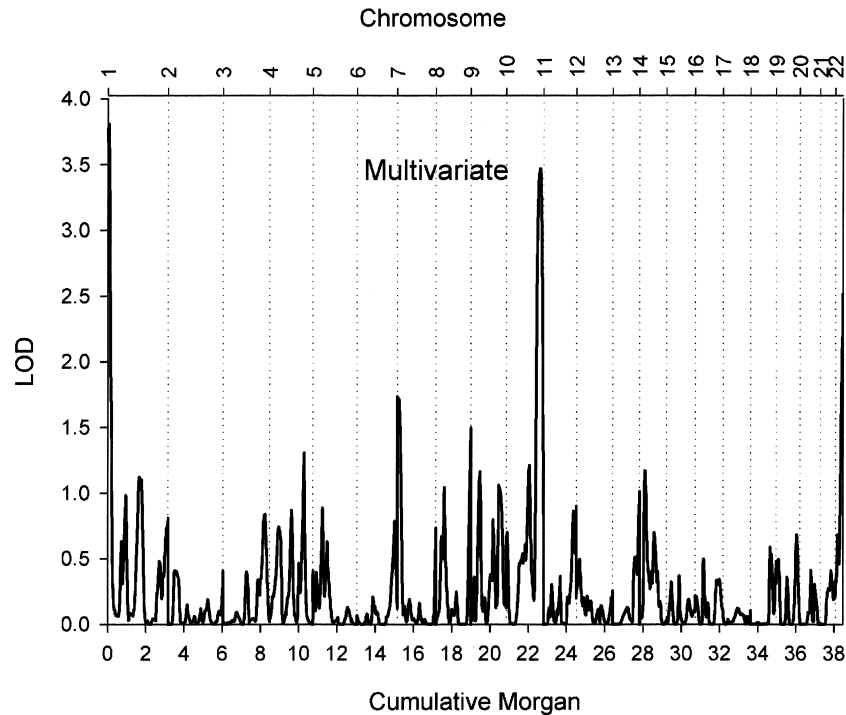
Figure 2 Multivariate, multipoint, variance-components linkage of the 22 autosomes in 270 sib pairs. Linkage results combine the four periods, each adjusted for mean effects of sex and age on respiration rate. The *X*-axis plots genetic distance in cM (Haldane), and the *Y*-axis represents the adjusted LOD score. The adjusted LOD score is determined as the 1-df LOD score required to give the same *P* value as the multivariate LOD score and can be compared directly with the univariate LOD scores.

found for respiration rate during sleep, on chromosome 10 at ~182 cM from pter, between markers *D10S1222* and *D10S1248*. A second locus had a suggestive influence on sleeping respiration rate (LOD score = 2.36); this locus was located on chromosome 3 at ~217 cM from pter, between markers *D3S1262* and *D3S2398*. Two additional regions were deemed of interest, because they yielded a LOD score >1.5 for respiration rate measured during at least two periods of the day. For morning and afternoon respiration rate, a suggestive peak was found on chromosome 7, near marker *D7S3056*. For evening and night respiration rate, suggestive peaks were found on chromosome 22; the peak for the evening respiration rate (LOD score = 2.75) was located at ~39 cM from pter, between markers *D22S689* and *D22S683*, and the peak for nighttime respiration rate (LOD score = 1.59) directly flanked it at ~48 cM from pter, at marker *D22S445*. For the four regions with the strongest evidence of linkage, LOD scores for each of the periods are displayed in table 2. A multivariate analysis using all four periods confirmed the potential importance of these four loci (fig. 2), which are plotted in more detail in figure 3. The high LOD score on chromosome 1 did not have a flanking marker at the telomeric end and was considered to be an artifact.

Obstructive sleep apnea and obesity are highly co-morbid, and the correlation between BMI and measures of sleep apnea has been shown to be attributable to genetic factors (Palmer et al. 2003). The correlations between respiration rate and BMI in our sample were all positive and significant (morning, 0.16; afternoon, 0.21; evening, 0.22; and night, 0.12; adjusted for age). The increased respiratory frequency with high BMI may reflect compensatory efforts against hypoventilation due to reduced chest-wall compliance or reduced nasopharyngeal caliber due to fat deposition in upper-airway tissues. To test this idea, we repeated all of the analyses described above with BMI as an additional covariate. Only neglible impact on the linkage signal was found, and the LOD scores for the four main loci (fig. 3) were essentially unchanged. Hence, linkage at these loci reflects direct genetic effects on respiratory regulation rather than indirect effects of genes influencing obesity.

Because respiration rate recorded during sleep showed the strongest linkage signals, the three loci with LOD scores >1.5 during sleep (on chromosomes 3 [217 cM], 10 [182 cM], and 22 [48 cM]) were modeled simultaneously as random effects in a three-loci linkage analysis. This simultaneous analysis is more robust to the well-known overestimation of variance attributable to mark-

A Chromosome 3

B Chromosome 7

C Chromosome 10

D Chromosome 22

Morning
Afternoon
Evening
Night
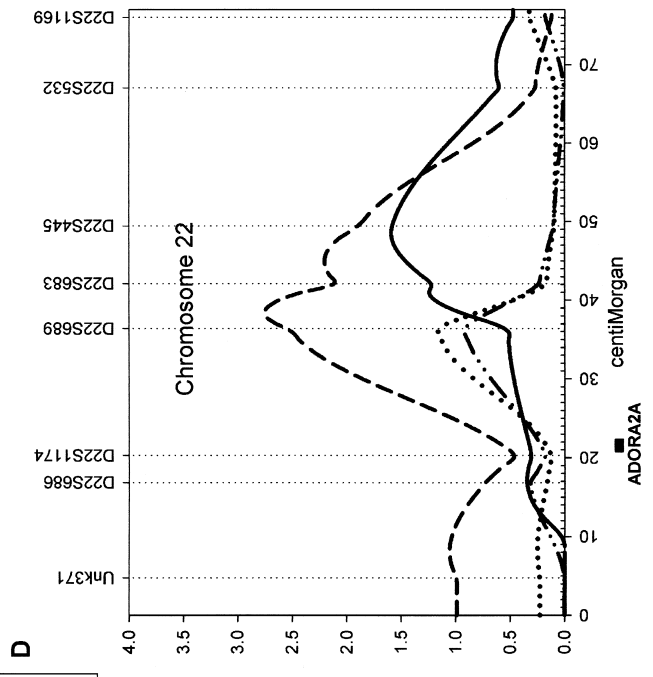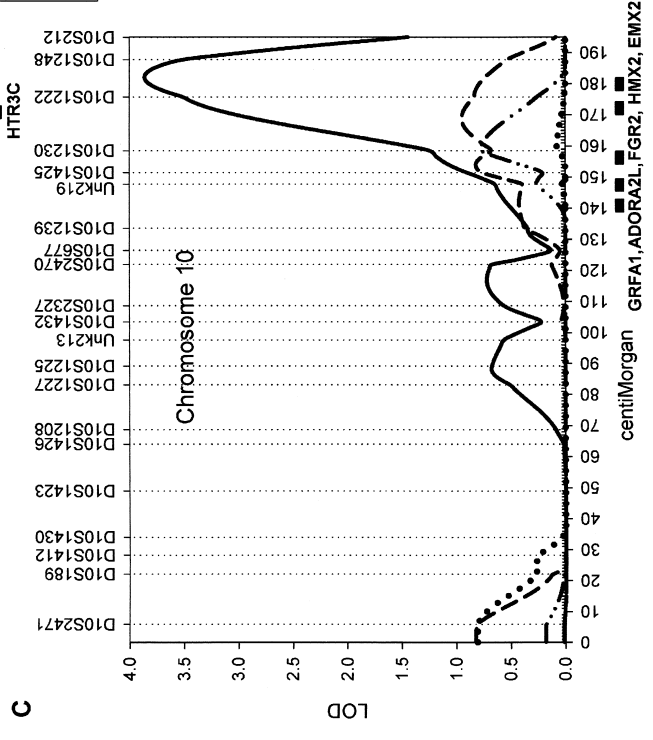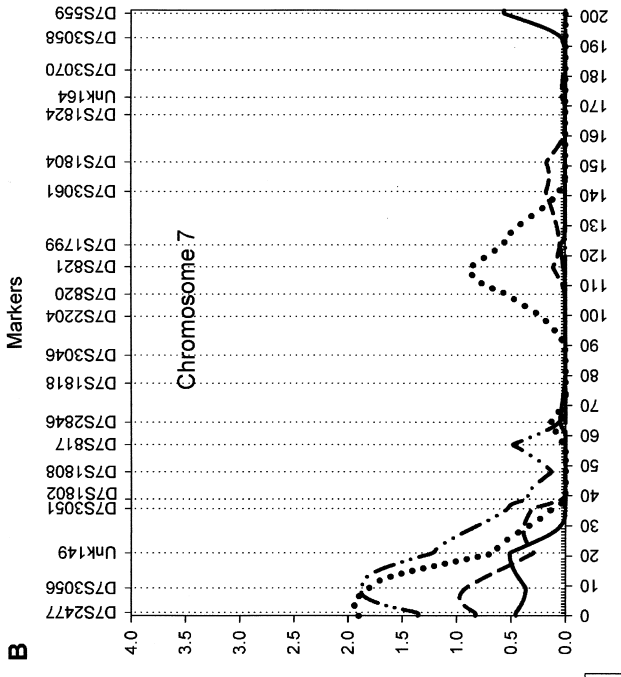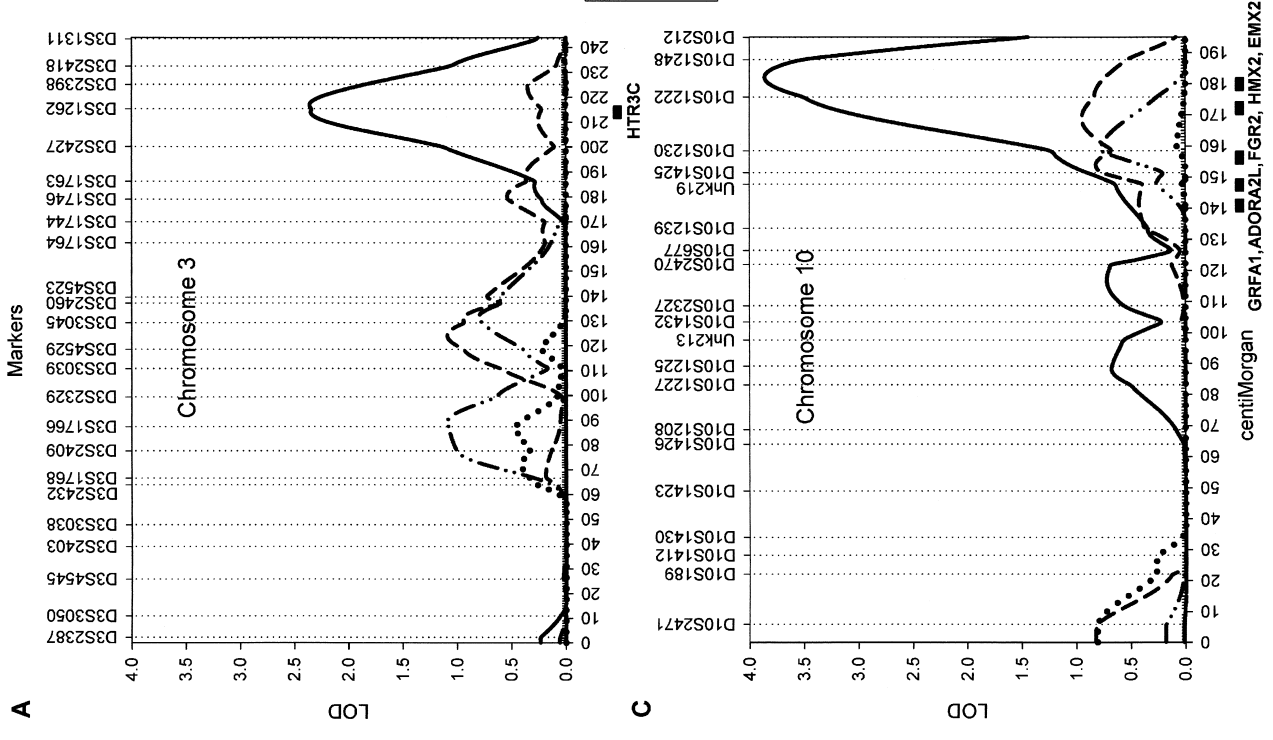
ers in linkage analyses. In a single-locus analysis, the QTL effects may be overestimated and together sum to a percentage of explained genetic variance >100% (Göring et al. 2001). The three-locus analysis constrains the sum of the QTL variances to be not >100%. Table 3 shows a significant LOD score for all three loci simultaneously. For chromosome 22, the evidence of linkage disappeared in the multilocus analysis, but note that, for this marker, the largest linkage was found in the evening, not during sleep. For chromosomes 3 and 10, the evidence weakened somewhat in comparison with the single-locus analyses. In models that included the locus on either chromosome 3 or chromosome 10, however, the amount of genetic variance attributable to these loci was highly comparable to that found in the single-locus analyses. There is a slight discrepancy between the χ^2 of a model leaving out three loci simultaneously and the summed χ^2 of the models leaving them out one at a time, suggesting an interaction between loci.

Discussion

Using ambulatory recording of the thorax impedance, we obtained 24-h respiration rates in healthy human subjects under natural conditions. Four genomic regions were identified as having a high likelihood of harboring loci that influence respiration rate. Linkage of a locus on chromosome 10q to nighttime respiration rate exceeded the Lander-Kruglyak threshold for significance (Lander and Kruglyak 1995). A second locus on chromosome 3q was also suggestively linked to nighttime respiration, as was a third locus on chromosome 22q. A simultaneous three-locus analysis confirmed the importance of the 3q and 10q loci for respiration rate during sleep. The generally higher LOD scores for nighttime respiration may reflect the increased power of the genome scan with an increase in the heritability of respiration rate. In a recent twin family study (H. M. Kupper, G. Willemsen, D. Posthuma, D. De Boer, D. I. Boomsma, E. J. C. de Geus, unpublished data), heritability of respiration rate was found to be moderate during the daytime (41%–50%) but to sharply increase at night (81%) through a decrease in environmental variance coupled to a strong increase in genetic variance. This shift in genetic architecture suggests that respiration rate is under more genetic control during sleep than during awake periods. Neurobiologically, this makes good sense. Transcription of a number of genes appears to be selectively increased during sleep (Mackiewicz and Pack 2003). In

addition, many environmental factors (speech, chewing, postural changes, and physical activity) impact respiration during the daytime, whereas, during sleep, respiratory frequency will be a more pure reflection of intrinsic rhythmogenesis by the brain stem.

Human and Animal Studies on the Genetics of Respiration

Several lines of evidence support an influence for genetic factors on respiratory control in humans. Most have focused on respiration during sleep because of its clinical relevance for sleep disorders, most prominently for obstructive sleep apnea (Gaultier et al. 2003; Palmer and Redline 2003). Attempts to find genes for respiration, therefore, have focused largely on the apnea-hypopnea index, the primary measure of obstructive sleep apnea. So far, candidate-gene association studies for apneic breathing have had limited success (Redline and Tishler 2000; Kadotani et al. 2001). In an authoritative review, Palmer and Redline (2003, table 2) compiled a list of the most plausible candidate genes for obstructive sleep apnea. None of these appeared to lie in the vicinity of the regions of (suggestive) linkage we found. In addition, our regions did not overlap with two loci (at 2p and 19p) for obstructive sleep apnea that were identified in a whole-genome scan of 66 pedigrees of American European origin (Palmer et al. 2003).

Congenital central hypoventilation syndrome (CCHS [MIM 209880]) is a second sleep disorder that may provide clues to positional candidates in the regions of our linkage peaks (Gaultier et al. 2003, 2004). This syndrome is characterized by deficient autonomic control over respiration and is hypothesized to account for some cases of sudden infant death syndrome (Gozal 1998). CCHS is caused by mutations of genes in the ret and endothelin pathways (see table 1 in Gaultier et al. 2004). The importance of some of these genes for respiration frequency has been confirmed in mice with loss-of-function mutations. Homozygous and heterozygous knock-outs of *gdnf*, *mash-1*, and *bdnf* all significantly affected resting respiratory frequency (see table 2 in Gaultier et al. 2004). The human gene corresponding to one of these genes, the glial cell line–derived neurotrophic factor (GDNF) family receptor alpha-1 gene (*GRFA1* [MIM 601496]), is mapped at ~5 Mb before the start of our region on chromosome 10. Gene expression profiling has suggested deviations in GRFA1 receptor regulation in Hirschsprung disease (MIM 142623), a condition that

Figure 3 Best evidence of linkage to ambulatory respiration rate on chromosomes 3 *(A)*, 7 *(B)*, 10 *(C)*, and 22 *(D)*. Linkage results are presented for each of the four periods and are adjusted for mean effects of sex and age on respiration rate. The *X*-axis plots genetic distance in cM (Haldane), and the *Y*-axis represents the multipoint variance-components LOD score. Markers are arrayed in map order along the top of each plot.

Table 3

Simultaneous Linkage Analysis of Three Loci with a Single-Locus LOD Score >1.5 for Respiration Rate during Sleep

		% Contribution to Variance for							
Model	Significance Test for	A	E	Chr 3	Chr 10	Chr 22	Δdf	χ^2	LOD
A+E+Chr 3, 10, and 22	...	0	11	33	56	0	...	...	...
A+E+Chr 10 and 22	Chr 3	10	20	...	60	9	1	9.02	1.96
A+E+Chr 3 and 22	Chr 10	9	23	40	...	28	1	12.91	2.81
A+E+Chr 3 and 10	Chr 22	0	11	33	56	...	1	0	0
A+E	Chr 3, 10, and 22	81	19	...	...	...	3	27.08	4.49[a]

Note.—The percentage contribution to the variance in respiration rate is given for background genetic influences (A), environmental influences (E), and the three loci on chromosomes 3 (217 cM), 10 (182 cM), and 22 (48 cM). The significance of the contribution of each locus separately and all three loci together derives from the contrast of the fit of the model with these loci (model row 1) and the fit of models without one (model rows 2–4) or all (model row 5) of these loci. Chr = chromosome(s); Δdf = change in degrees of freedom.

[a] Adjusted LOD score, determined as the 1-df LOD score required to give the same *P* value as the multilocus LOD score.

is comorbid with CCHS (Iwashita et al. 2003), and at least one patient with CCHS showed a mutation in *GFRA1* (Sasaki et al. 2003). Its proximity to our peak LOD score at 10q suggests that polymorphisms in this gene may affect variation in nighttime respiration rate in healthy subjects.

Animal models of respiratory rhythmogenesis and regulatory input to the brain stem have provided a number of further candidate genes. Hox paralogs and hox-regulating genes (e.g., *hoxa, kreisler/mafB, c-jun,* and *Krox20*) are involved in the primordial rhombomeric organization of the hindbrain. Knockout mutations in these genes resulted in phenotypes of decreased or increased respiratory frequency, compared with that of the wild type (Jacquin et al. 1996; Shirasawa et al. 2000; Domínguez del Toro et al. 2001; Chatonnet et al. 2002). Mice engineered to lack the bZIP transcriptional regulator gene *MafB,* prominently expressed in the pre-Bötzinger complex, were shown to completely lack development of critical rhythm-generating neurons in the brain stem (Blanchi et al. 2003). Although none of these "rodent respiratory genes" had syntenic human genes in any of our four regions of linkage, at least two other homeobox genes, *HMX2* (MIM 600647) and *EMX2* (MIM 600035), with as-yet unknown relation to respiration, were found under our best linkage peak on 10q.

Homeobox genes have also been prominently included in the list of possible candidate genes for obstructive sleep apnea (Palmer and Redline 2003), because they can have an effect on craniofacial form and upper-airway anatomy. The >100 craniofacial malformation ("craniosynostosis") syndromes may well be regarded as the third "disorder" that can affect ventilation. Craniofacial development from skeletogenic differentiation of the cranial neural crest is governed almost entirely by the fibroblast growth factors (Wilkie and Morriss-Kay 2001). A major role in craniosynostosis syndromes is suggested for fibroblast growth factor receptor 2 gene

(*FGFR2* [MIM 176943]), which is directly under our best linkage peak at 10q26. Deviant respiratory control has frequently been reported in craniosynostosis syndromes, often severe enough to require surgical correction or prolonged continuous positive airway pressure therapy (Gonsalez et al. 1996; Perkins et al. 1997). Milder nonmorbid mutations in *FGFR2* may well affect population variation in craniofacial build and basal respiration rate.

Positional Candidates

In a second strategy to confirm our linkage-peak results, we scanned the Ensembl human genome map (version 19.34b.2 of NCBI assembly 34 [July 2004 freeze]; see Ensemble Web site) for genes in these regions that could be plausibly linked to respiratory behavior. "Broad" peaks were used, spanning between the markers that defined the upstroke and incisura of the LOD score peaks (*D3S2427–D3S2418, D7S2477–D7S3051, D10S1230–D10S212,* and *D22S1174–D22S532*). By necessity, successful recognition of potentially relevant genes in the Ensembl-generated lists of genes in the linkage regions is limited by current understanding of the molecular biology of respiration. At least five positional candidate genes, however, could be plausibly connected to the regulation of nighttime respiratory frequency.

Adenosine and its analogues have been shown to increase respiratory ventilation in a dose-dependent manner (Monteiro and Ribeiro 1987) and to modulate the incidence of sleep apneas in rats (Monti et al. 1995). In a sheep model of fetal breathing, A_1 receptors were found to tonically inhibit respiratory drive, A_{2A} receptors to tonically inhibit REM sleep, and both A_1 and A_{2A} receptors to mediate the depressant effects of adenosine on REM sleep and breathing (Koos et al. 2001). Adenosine mediates its effects through four receptor subtypes: the A_1, A_{2A}, A_{2B}, and A_3 receptors (Fredholm et al. 1994). MacCollin et al. (1994) localized the *ADORA2A* gene

(MIM 102776) to chromosome 22q11.23, just outside our region on 22q that showed linkage to respiration rate during the evening and sleep. At 10q25.3-q26.3 (*ADORA2L* [MIM 102777]), another gene for an A_2 adenosine receptor subtype is suspected, the function of which is currently unknown. Given the convergence of the loci at chromosomes 10 and 22 in a single biological pathway and the importance of adenosine in respiration, we suggest that *ADORA2A* and *ADORA2L* may be promising positional candidate genes for respiration rate.

Serotonin and serotonergic drugs have significant effects on respiration, and serotonin has been implicated in the pathogenesis of sleep disorders (Richerson 2004). Serotonergic neurons in the Raphé nucleus act as chemoreceptors and enhance respiratory rhythm generation in the preBötzinger complex. These effects have been shown to be mediated through serotonin receptors type 1A, 2A , 4A, and 7 (Richter et al. 2003; Richerson 2004). However, a role for serotonin type 3 (5-HT$_3$) receptors can also be assumed. Serotonin antagonists selective for the 5-HT$_3$ receptor suppress sleep-related central apneas in rats (Radulovacki et al. 1998) and obstructive sleep apnea in the English bulldog (Veasey et al. 2001). A recently identified cluster of three novel serotonin type 3 receptor genes (*HTR3C, HTR3D,* and *HTR3E*) is localized at 3q27 (Karnovsky et al. 2003), directly in our area of linkage. Comparative expression analysis suggested that *HTR3D* and *HTR3E* expression were limited to colon, kidney, liver, and intestine, whereas the *HTR3C* gene is widely expressed in many tissues, including the brain (Niesler et al. 2003). These findings lead us to suggest that *HTR3C,* a gene homologous to 5-HT$_{3A}$ and 5-HT$_{3B}$ receptors, qualifies as a potential candidate gene for respiration rate.

In summary, evidence of linkage was found for respiration rate during sleep at 3q27, 7p22, 10q26, and 22q12. Strongest evidence of linkage was found between markers *D10S1222* and *D10S1248* on chromosome 10. From the Ensembl database, we identified *GFRA1, ADORA2L, FGR2, EMX2,* and *HMX2* as biologically plausible candidate genes harbored by this linkage region. Further candidates suggested by our linkage findings are the *ADORA2A* adenosine receptor gene on 22q and the *HTRC3* serotonin receptor gene at 3q. Identification of the genetic variation influencing human respiratory phenotypes would serve an important clinical purpose. It could increase our understanding of the molecular and cellular bases of disorders of rhythmogenesis such as sleep apnea, unexplained stillbirth, and certain cases of sudden infant death syndrome.

Acknowledgments

Electronic-Database Information

The URLs for data presented herein are as follows:

Ensembl, http://www.ensembl.org/ (for human genome map version 19.34b.2 of NCBI assembly 34 [July 2004 freeze])

Marshfield Center for Medical Genetics, http://www.marshfieldclinic.org/research/genetics/

Mx software, http://www.vcu.edu/mx

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for CCHS, *GRFA1,* Hirschsprung disease, *HMX2, EMX2, FGFR2, ADORA2A,* and *ADORA2L*)

References

Abecasis GR, Cherny SS, Cookson WO, Cardon LR (2001) GRR: graphical representation of relationship errors. Bioinformatics 17:742–743

——— (2002) Merlin—rapid analysis of dense genetic maps using sparse gene flow trees. Nat Genet 30:97–101

American Academy of Sleep Medicine Task Force (1999) Sleep-related breathing disorders in adults: recommendations for syndrome definition and measurement techniques in clinical research. Sleep 22:667–689

Blanchi B, Kelly LM, Viemari JC, Lafon I, Burnet H, Bévengut M, Tillmanns S, Daniel L, Graf T, Hilaire G, Sieweke MH (2003) MafB deficiency causes defective respiratory rhythmogenesis and fatal central apnea at birth. Nat Neurosci 6:1091–1100

Boomsma DI, Beem AL, van den Berg M, Dolan CV, Koopmans JR, Vink JM, de Geus EJ, Slagboom PE (2000) Netherlands twin family study of anxious depression (NETSAD). Twin Res 3:323–334

Boomsma DI, Vink JM, Van Beijsterveldt TC, de Geus EJ, Beem AL, Mulder EJ, Derks EM, Riese H, Willemsen GA, Bartels M, van den Berg M, Kupper NH, Polderman TJ, Posthuma D, Rietveld MJ, Stubbe JH, Knol LI, Stroet T, van Baal GC (2002) Netherlands Twin Register: a focus on longitudinal research. Twin Res 5:401–406

Broman KW, Murray JC, Sheffield VC, White RL, Weber JL (1998) Comprehensive human genetic maps: individual and sex-specific variation in recombination. Am J Hum Genet 63:861–869

Chatonnet F, Thoby-Brisson M, Abadie V, Domínguez del Toro E, Champagnat J, Fortin G (2002) Early development of respiratory rhythm generation in mouse and chick. Respir Physiol Neurobiol 131:5–13

de Geus EJC, Van Doornen LJP (1996) Ambulatory assessment of parasympathetic/sympathetic balance by impedance cardiography. In: Fahrenberg J, Myrtek M (eds) Computer-assisted psychological and psychophysiological methods in

monitoring and field studies. Hogrefe and Huber Publishers, Seattle, pp 141–163

de Geus EJC, Willemsen AHM, Klaver CH, Van Doornen LJ (1995) Ambulatory measurement of respiratory sinus arrhythmia and respiration rate. Biol Psychol 41:205–227

Domínguez del Toro E, Borday V, Davenne M, Neun R, Rijli FM, Champagnat J (2001) Generation of a novel functional neuronal circuit in *Hoxa1* mutant mice. J Neurosci 21:5637–5642

Feldman JL, Mitchell GS, Nattie EE (2003) Breathing: rhythmicity, plasticity, chemosensitivity. Annu Rev Neurosci 26:239–266

Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, Leff P, Williams M (1994) Nomenclature and classification of purinoceptors. Pharmacol Rev 46:143–156

Gaultier C, Amiel J, Dauger S, Trang H, Lyonnet S, Gallego J, Simonneau M (2004) Genetics and early disturbances of breathing control. Pediatr Res 55:729–733

Gaultier C, Dauger S, Simonneau M, Gallego J (2003) Genes modulating chemical breathing control: lessons from mutant animals. Respir Physiol Neurobiol 136:105–114

Gonsalez S, Thompson D, Hayward R, Lane R (1996) Treatment of obstructive sleep apnoea using nasal CPAP in children with craniofacial dysostoses. Childs Nerv Syst 12:713–719

Göring HH, Terwilliger JD, Blangero J (2001) Large upward bias in estimation of locus-specific effects from genomewide scans. Am J Hum Genet 69:1357–1369

Gozal D (1998) Congenital central hypoventilation syndrome: an update. Pediatr Pulmonol 26:273–282

Hanly PJ (1992) Mechanisms and management of central sleep apnea. Lung 170:1–17

Hilaire G, Pásaro R (2003) Genesis and control of the respiratory rhythm in adult mammals. News Physiol Sci 18:23–28

Iwashita T, Kruger GM, Pardal R, Kiel MJ, Morrison SJ (2003) Hirschsprung disease is linked to defects in neural crest stem cell function. Science 301:972–976

Jacquin TD, Borday V, Schneider-Maunoury S, Topilko P, Ghilini G, Kato F, Charnay P, Champagnat J (1996) Reorganization of pontine rhythmogenic neuronal networks in *Krox-20* knockout mice. Neuron 17:747–758

Kadotani H, Kadotani T, Young T, Peppard PE, Finn L, Colrain IM, Murphy GM Jr, Mignot E (2001) Association between apolipoprotein E $\epsilon4$ and sleep-disordered breathing in adults. JAMA 285:2888–2890

Karnovsky AM, Gotow LF, McKinley DD, Piechan JL, Ruble CL, Mills CJ, Schellin KA, Slightom JL, Fitzgerald LR, Benjamin CW, Roberds SL (2003) A cluster of novel serotonin receptor 3-like genes on human chromosome 3. Gene 319:137–148

Kong A, Gudbjartsson DF, Sainz J, Jonsdottir GM, Gudjonsson SA, Richardsson B, Sigurdardottir S, Barnard J, Hallbeck B, Masson G, Shlien A, Palsson ST, Frigge ML, Thorgeirsson TE, Gulcher JR, Stefansson K (2002) A high-resolution recombination map of the human genome. Nat Genet 31:241–247

Koos BJ, Maeda T, Jan C (2001) Adenosine A_1 and A_{2A} re-

ceptors modulate sleep state and breathing in fetal sheep. J Appl Physiol 91:343–350

Lander E, Kruglyak L (1995) Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. Nat Genet 11:241–247

Lystig TC (2003) Adjusted *P* values from genome-wide scans. Genetics 164:1683–1687

MacCollin M, Peterfreund R, MacDonald M, Fink JS, Gusella J (1994) Mapping of a human A2a adenosine receptor (ADORA2) to chromosome 22. Genomics 20:332–333

Mackiewicz M, Pack AI (2003) Functional genomics of sleep. Respir Physiol Neurobiol 135:207–220

Monteiro EC, Ribeiro JA (1987) Ventilatory effects of adenosine mediated by carotid body chemoreceptors in the rat. Naunyn Schmiedebergs Arch Pharmacol 335:143–148

Monti D, Carley DW, Radulovacki M (1995) Adenosine analogues modulate the incidence of sleep apneas in rats. Pharmacol Biochem Behav 51:125–131

Niesler B, Frank B, Kapeller J, Rappold GA (2003) Cloning, physical mapping and expression analysis of the human 5-HT_3 serotonin receptor-like genes *HTR3C, HTR3D* and *HTR3E.* Gene 310:101–111

Palmer LJ, Buxbaum SG, Larkin E, Patel SR, Elston RC, Tishler PV, Redline S (2003) A whole-genome scan for obstructive sleep apnea and obesity. Am J Hum Genet 72:340–350

Palmer LJ, Redline S (2003) Genomic approaches to understanding obstructive sleep apnea. Respir Physiol Neurobiol 135:187–205

Perkins JA, Sie KC, Milczuk H, Richardson MA (1997) Airway management in children with craniofacial anomalies. Cleft Palate Craniofac J 34:135–140

Radulovacki M, Trbovic SM, Carley DW (1998) Serotonin 5-HT_3-receptor antagonist GR 38032F suppresses sleep apneas in rats. Sleep 21:131–136

Redline S, Tishler PV (2000) The genetics of sleep apnea. Sleep Med Rev 4:583–602

Rekling JC, Feldman JL (1998) PreBötzinger complex and pacemaker neurons: hypothesized site and kernel for respiratory rhythm generation. Annu Rev Physiol 60:385–405

Richerson GB (2004) Serotonergic neurons as carbon dioxide sensors that maintain pH homeostasis. Nat Rev Neurosci 5:449–461

Richter DW, Manzke T, Wilken B, Ponimaskin E (2003) Serotonin receptors: guardians of stable breathing. Trends Mol Med 9:542–548

Richter DW, Spyer KM (2001) Studying rhythmogenesis of breathing: comparison of in vivo and in vitro models. Trends Neurosci 24:464–472

Sasaki A, Kanai M, Kijima K, Akaba K, Hashimoto M, Hasegawa H, Otaki S, Koizumi T, Kusuda S, Ogawa Y, Tuchiya K, Yamamoto W, Nakamura T, Hayasaka K (2003) Molecular analysis of congenital central hypoventilation syndrome. Hum Genet 114:22–26

Schaffer AA (1996) Faster linkage analysis computations for pedigrees with loops or unused alleles. Hum Hered 46:226–235

Self SG, Liang K-Y (1987) Asymptotic properties of maximum likelihood estimators and likelihood ratio tests under nonstandard conditions. J Am Stat Assoc 82:605–610

Sham P (1998) Statistics in human genetics. Arnold Publishers, London

Sham PC, Purcell S, Cherny SS, Abecasis GR (2002) Powerful regression-based quantitative-trait linkage analysis of general pedigrees. Am J Hum Genet 71:238–253

Shirasawa S, Arata A, Onimaru H, Roth KA, Brown GA, Horning S, Arata S, Okumura K, Sasazuki T, Korsmeyer SJ (2000) *Rnx* deficiency results in congenital central hypoventilation. Nat Genet 24:287–290

Veasey SC, Chachkes J, Fenik P, Hendricks JC (2001) The effects of ondansetron on sleep-disordered breathing in the English bulldog. Sleep 24:155–160

Wilkie AO, Morriss-Kay GM (2001) Genetics of craniofacial development and malformation. Nat Rev Genet 2:458–468

Wolk R, Kara T, Somers VK (2003) Sleep-disordered breathing and cardiovascular disease. Circulation 108:9–12

Yuan B, Vaske D, Weber JL, Beck J, Sheffield VC (1997) Improved set of short-tandem-repeat polymorphisms for screening the human genome. Am J Hum Genet 60:459–460