Brief Communication

Absence of *Ndn*, Encoding the Prader-Willi Syndrome-Deleted Gene *necdin*, Results in Congenital Deficiency of Central Respiratory Drive in Neonatal Mice

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necdin (_Ndn_) is one of a cluster of genes deleted in the neurodevelopmental disorder Prader-Willi syndrome. _necdin_ is upregulated during neuronal differentiation and is thought to play a role in cell cycle arrest in terminally differentiated neurons. Most _necdin_-deficient _Ndn_^{tm2Stw} mutant pups carrying a targeted replacement of _Ndn_ with a _lacZ_ reporter gene die in the neonatal period of apparent respiratory insufficiency. We now demonstrate that the defect can be explained by abnormal neuronal activity within the putative respiratory rhythm-generating center, the pre-Bötzinger complex. Specifically, the rhythm is unstable with prolonged periods of depression of respiratory rhythmogenesis. These observations suggest that the developing respiratory center is particularly sensitive to loss of _necdin_ activity and may reflect abnormalities of respiratory rhythm-generating neurons or conditioning neuromodulatory drive. We propose that _necdin_ deficiency may contribute to observed respiratory abnormalities in individuals with Prader-Willi syndrome through a similar suppression of central respiratory drive.

Key words: **Prader-Willi; apnea; _necdin_; medulla; breathing; newborn**

Introduction

necdin (neurally differentiated embryonal carcinoma-cell derived factor) is one of four known protein-coding genes that are deficient in people with Prader-Willi syndrome (PWS) (Jay et al., 1997; MacDonald and Wevrick, 1997; Sutcliffe et al., 1997). PWS is a developmental neurobehavioral disorder (Online Mendelian Inheritance in Man entry number 176270) that occurs sporadically at a frequency of ~1 in 15,000 (Holm et al., 1993). The major manifestations of PWS include neonatal hypotonia and failure to thrive, followed by childhood-onset developmental delay and obesity. Infants with PWS have significant respiratory abnormalities, including sleep-related central and obstructive apneas and reduced response to changes in oxygen and CO_2 levels (Arens et al., 1994; Clift et al., 1994; Gozal et al., 1994; Wharton and Loechner, 1996; Schluter et al., 1997; Menendez, 1999; Manni et al., 2001; Nixon and Brouillette, 2002). A subset of genes in the region deleted in PWS, including the *NDN* gene encoding *necdin*, are active only on the paternally inherited allele and silenced by imprinting on the maternal allele (Nicholls, 2000). The relative contribution of the loss of each gene to the complex PWS phenotype is as yet unknown, and there are no known cases of PWS attributable to deficiency of only one protein-encoding gene.

necdin was originally identified as a gene upregulated during the retinoic acid-induced differentiation of postnatal day 19 embryonic carcinoma cells into neurons (Maruyama et al., 1991). The expression of *necdin* in mouse development mirrors the cultured cell system, because *necdin* is expressed in many but not all postdifferentiation stage neurons. *necdin* is a member of the *MAGE* (melanoma antigen-encoding gene)/*necdin* gene family that also includes *MAGEL2*, also deficient in PWS (Boccaccio et al., 1999; Lee et al., 2000).

Three *necdin*-deficient mouse strains were independently generated by homologous recombination in embryonic stem cells (Gerard et al., 1999; Tsai et al., 1999; Muscatelli et al., 2000). In all three strains, heterozygous mice that inherit the mutated allele maternally are indistinguishable from their wild-type littermates, because of imprinting that normally silences the maternal allele. Two *necdin*-deficient mouse strains carrying a paternally inherited *Ndn* deletion allele are affected by postnatal lethality. Deficiency of *necdin* in these mice causes neonatal respiratory distress that is usually fatal, and surviving mice exhibit mildly abnormal behavior (Gerard et al., 1999; Muscatelli et al., 2000). In the original targeted allele of Gérard et al. (1999), there is ~70% lethality in the first 30 postnatal hours. Deletion of the phosphoglycerate kinase-neo cassette present in the original targeted allele increased the lethality to 98% in the *Ndn*^{tm2Stw} *necdin*-

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deficient strain (Gerard et al., 1999), possibly because of an effect on nearby genes of the neomycin promoter.

Functional defects of the lungs, respiratory musculature, chemoreception, or central neural control mechanisms could account for the respiratory distress phenotype. In this study, we used *in vitro* preparations to assess the respiratory neuronal activity at multiple sites along the central neuraxis. Specifically, we test the hypothesis that the hypoventilation results from a defective central respiratory drive in *necdin*-deficient mice.

Materials and Methods

Mouse breeding and genotyping. Procedures for animal care were approved by the Animal Welfare Committee at the University of Alberta. Ndn^{tm2Stw} *necdin*-deficient mice were bred through the maternal line with C57BL/6J male mice. Male offspring carrying a maternally inherited Ndn^{tm2Stw} are phenotypically normal and were bred to C57BL/6J females to produce experimental embryos and offspring. In these litters, one-half of the mice are wild type, and one-half carry a paternally inherited *necdin* deficiency and are functionally null. The timing of pregnancies was determined from the appearance of sperm plugs in the breeding cages [embryonic day 0.5 (E0.5)]. Identification of mutant offspring was performed by PCR genotyping with *lacZ* oligonucleotide primers (LACZ1942F, 5′GTGTCGTTGCTGCATAAACC; and LACZ2406R, 5′TCGTCTGCTCATCCATGACC) or by histochemical detection of spare tissue. For detection of β-galactosidase activity, tissue samples were fixed in cold 0.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 8. The samples were incubated in β-galactosidase stain until appropriate stain intensity was observed.

Brainstem–spinal cord preparations. Fetal mice (E18.5) were delivered from timed-pregnant mice anesthetized with halothane (1.25–1.5% delivered in 95% O_2 and 5% CO_2) and maintained at 37°C by radiant heat. Newborn mice were anesthetized by inhalation of metofane (2–3%). Embryos and newborns were decerebrated, and the brainstem–spinal cord with or without the ribcage and diaphragm muscle attached was dissected following procedures similar to those established for perinatal rats (Smith et al., 1990; Greer et al., 1992). The neuraxis was continuously perfused at 27 ± 1°C (perfusion rate of 5 ml/min; chamber volume of 1.5 ml) with mock CSF that contained the following (in mM): 128 NaCl, 3.0 KCl, 1.5 $CaCl_2$, 1.0 $MgSO_4$, 24 $NaHCO_3$, 0.5 NaH_2PO_4, and 30 D-glucose (equilibrated with 95%O_2–5%CO_2).

Medullary slice preparations. Details of the preparation have been described previously (Smith et al., 1991). Briefly, the brainstem–spinal cords isolated from perinatal mice as described above were pinned down, ventral surface upward, on a paraffin-coated block. The block was mounted in the vise of a vibratome bath (VT1000S; Leica, Nussloch, Germany). The brainstem was sectioned serially in the transverse plane starting from the rostral medulla to within ~150 μm of the rostral boundary of the pre-Bötzinger complex, as judged by the appearance of the inferior olive. A single transverse slice containing the pre-Bötzinger complex and more caudal reticular formation regions was then cut (~400 μm thick), transferred to a recording chamber, and pinned down onto a Sylgard elastomer. The medullary slice was continuously perfused in physiological solution similar to that used for the brainstem–spinal cord preparation except for the potassium concentration, which was increased to 9 mM to stimulate the spontaneous rhythmic respiratory motor discharge in the medullary slice (Smith et al., 1991).

Recording and analysis. Recordings of hypoglossal (XII) cranial nerve roots, cervical (C4) ventral roots, and diaphragm EMG were made with suction electrodes. Furthermore, suction electrodes were placed into XII nuclei and the pre-Bötzinger complex to record extracellular neuronal population discharge from medullary slice preparations. Signals were amplified, rectified, low-pass filtered, and recorded on a computer using an analog-to-digital converter (Digidata 1200; Axon Instruments, Foster City, CA) and data acquisition software (Axoscope; Axon Instruments). Mean values relative to control for the period and peak integrated amplitude of respiratory motoneuron discharge were calculated. Values given are means, SDs, and coefficients of variability (SD/mean). Statistical significance was tested using paired difference Student's *t* test; significance was accepted at p values <0.05.

Whole-cell recordings. Recording electrodes were fabricated from thin-wall borosilicate glass (1.5 mm external and 1.12 mm internal diameter; A-M Systems, Everett, WA). The pipette resistances were between 3 and 4 MΩ. The standard pipette solution contained the following (in mM): 130 potassium gluconate, 10 NaCl, 1 $CaCl_2$, 10 BAPTA, 10 HEPES, 5 Mg ATP, and 0.3 NaGTP, pH 7.3 with KOH. Whole-cell current-clamp recordings were initially established in the artificial CSF solution and performed with an NPI Electronics SEC05LX amplifier (NPI Electronics, Tamm, Germany). Liquid junction potentials were corrected before seal formation with the compensation circuitry of the patch-clamp amplifier. Data were digitized with an analog-to-digital interface (Digidata 1322a; Axon Instruments) and analyzed with the use of pClamp 8.0 (Axon Instruments).

RNA in situ *hybridization.* A cloned PCR product containing partial open reading frame and 3′ untranslated region of mouse *Ndn* (base positions 162–1235; GenBank accession number M80840) was used as a template for riboprobe synthesis. The digoxygenin-labeled RNA antisense riboprobe was synthesized using T7 RNA polymerase and DIG RNA labeling kit (Roche Products, Hertforshire, UK). Cryostat sections, 60 μm thick, were processed for *in situ* hybridization essentially as described previously (Wilkinson and Nieto, 1993). Processed sections were hybridized on slides at 68°C overnight. Post-hybridization washes were at 68°C with no ribonuclease A treatment. Levamisole (2 mM) was added to all subsequent steps. Slides were preblocked with 5% blocking reagent (Roche Products) before incubation with preabsorbed antibodies for 6 hr at room temperature.

Results

Respiratory rhythms are perturbed in Ndn^{tm2Stw} mutant newborn mice

In litters of newborn mice born to a heterozygous Ndn^{tm2Stw} male and wild-type female, we observed that a subset of pups gasped for air, turned cyanotic, and died over a postnatal time course of a few hours, as noted previously (Gerard et al., 1999; Muscatelli et al., 2000). Nudging the pups caused a transient increase in respiration and loss of cyanosis. Pups exhibiting normal ($n = 7$) and abnormal ($n = 8$) respiration were selected, and brainstem–spinal cord preparations were isolated within 20 min of birth. Among pups with abnormal breathing patterns *in vivo*, seven of eight failed to generate rhythmic motor bursts from cervical or hypoglossal nerve roots *in vitro*. The remaining pups generated a severely irregular rhythmic motor output. Subsequent genotyping confirmed that preparations with markedly perturbed respiratory rhythms were Ndn^{tm2Stw} mutants. Although these data were informative, the fact that newborns with respiratory dysfunction were hypoxic and stressed during the early postnatal period could have been a confounding factor. For instance, the central neural control mechanisms could have been compromised secondarily to a primary defect of lung function or peripheral respiratory afferent input. Therefore, we proceeded to assess the central drive in embryos delivered via cesarean section at E18.5.

Respiratory discharge in Ndn^{tm2Stw} mutant embryos at E18.5

Simultaneous suction electrode recordings of inspiratory motor discharge were made from diaphragm muscle and/or hypoglossal (XII) nerve roots in brainstem–spinal cord preparations with the ribcage and diaphragm attached. A total of 36 putative *necdin*-deficient (abnormal respiratory rhythm) and 22 wild-type E18.5 embryonic mice were subsequently selected for detailed analyses. In each case, postexperimental genotyping confirmed the identity of wild-type and Ndn^{tm2Stw} mutant mice. In Ndn^{tm2Stw} mutant mice, the rhythms were consistently irregular, with promi-

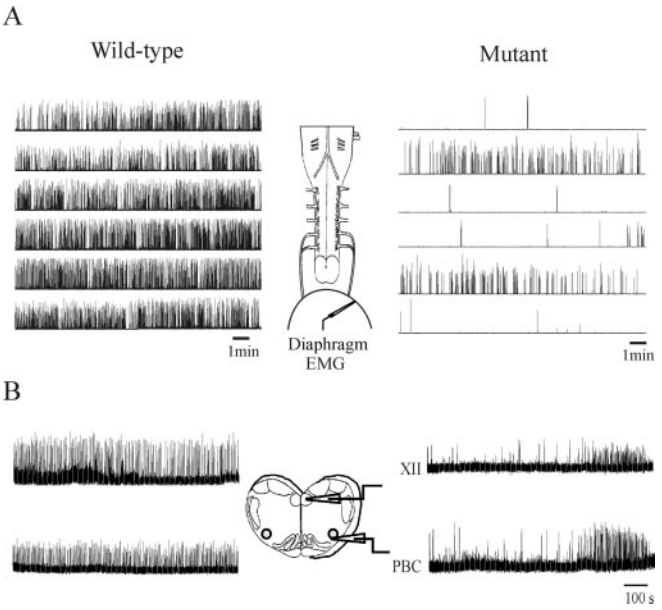

Figure 1. *necdin*-deficient *Ndn*^{tm2Stw} mice have irregular respiratory rhythms with prolonged periods of central apnea. *A*, Sample rectified and integrated suction electrode recordings of diaphragm EMG were made from brainstem–spinal cord–diaphragm preparations isolated from E18.5 wild-type (*left*) and *Ndn*^{tm2Stw} mutant (*right*) mice. Recordings of 80 min duration demonstrate the regularity of respiratory discharge frequency (~4–5 sec interspike interval) in wild-type preparations. In contrast, the respiratory frequency is very unstable in mutant preparations over time. *B*, Defects in respiratory rhythm are observed within the putative respiratory rhythm-generating center. Sample rectified and integrated suction electrode recordings were made from inspiratory neurons located in the pre-Bötzinger complex (*PBC*) and neurons within the hypoglossal (*XII*) nucleus in medullary slice preparations isolated from E18.5 wild-type (*left*) and *Ndn*^{tm2Stw} mutant (*right*) mice.

nent bouts of respiratory depression characterized by burst frequencies of one to three bursts per 10 min period and central apneas persisting for up to several minutes (Fig. 1*A*). The bouts of suppressed respiratory rhythmic discharge were interspersed with periods of inspiratory motor bursts close to frequencies observed in wild-type preparations (Table 1). There were no marked differences in the amplitude or duration of inspiratory bursts. These recordings demonstrate that the defect in rhythmic motor discharge is present in both cranial and spinal motoneuron populations.

We selected 18 of the *Ndn*^{tm2Stw} mutant mouse preparations and removed the ribcage and diaphragm musculature. The rhythmic discharge pattern recorded from the fourth cervical root was similar to that recorded from the diaphragm EMG in 7 of 18 preparations. The other 11 *Ndn*^{tm2Stw} mutant mice failed to produce any respiratory motor output from cervical or hypoglossal nerve roots during removal of the ribcage and diaphragm musculature. Presumably, the threshold excitation necessary to achieve rhythmic motor output in these mutants was only achieved with the intact musculature and associated afferent input.

Medullary slice preparations from *Ndn*^{tm2Stw} mutant embryos at E18.5

We recorded rhythmic respiratory discharge from the hypoglossal (XII) motoneuron pool in medullary slice preparations isolated from *Ndn*^{tm2Stw} mutant and wild-type mice (Fig. 1*B*). The rhythmic neuronal discharge was irregular in all *Ndn*^{tm2Stw} mutant mice ($n = 10$) but robust and regular in all wild-type ($n = 8$) preparations. There were no cases in which *Ndn*^{tm2Stw} medullary slice preparations failed to generate some sort of rhythmic motor output. The elevated extracellular K^+ (9 mM) provided sufficient excitatory drive to respiratory neuronal populations to reach a threshold for generating a rhythmic, albeit irregular, pattern.

We next determined whether or not the abnormal respiratory rhythm was present within the pre-Bötzinger complex. Suction electrode recordings of population neuronal activity were performed in the region of the pre-Bötzinger complex. The rhythmic discharge of neurons within the pre-Bötzinger complex of mutant preparations had the same abnormal characteristics as the XII motor discharge (Fig. 1*B*, Table 1). Next, whole-cell patch-clamp recordings of inspiratory neurons within the pre-Bötzinger complex were performed. As illustrated in Figure 2, the neurons fired with an irregular rhythm with prolonged periods of suppressed rhythmogenesis. The resting membrane potential of inspiratory neurons became more depolarized during epochs of increased respiratory rhythmic frequency. There were also bouts of longer-duration bursting activity that is not of respiratory origin (Greer et al., 1992).

necdin mRNA expression in the medulla

Previous investigations of *necdin* gene expression by RNA *in situ* hybridization or immunohistochemistry had focused on the cerebrum, cerebellum, and the hypothalamus (Uetsuki et al., 1996; Niinobe et al., 2000). Expression of the *Ndn*^{tm2Stw} *lacZ* reporter gene had been noted in the medulla, spinal cord, and dorsal root ganglia in E17 embryos (Gerard et al., 1999). We examined the expression of *necdin* by RNA *in situ* hybridization in wild-type medullary sections at E15.5, when respiratory activity commences, and E18.5, the stage used for electrophysiological recordings. This experiment was to determine whether only subpopulations of neurons express *necdin*, as observed in other structures of the nervous system. *necdin* expression was evident in the ventrolateral medulla in which the respiratory rhythm generator is located, but levels here were not significantly different from in other medullary regions (Fig. 3).

Discussion

necdin-deficient *Ndn*^{tm2Stw} newborn mice hypoventilate, rapidly turn cyanotic, and die. We sought to assess centrally generated respiratory rhythmogenesis and drive transmission in isolation from other aspects of the respiratory system (e.g., lung function and peripheral afferent feedback). The brainstem–spinal cord–diaphragm preparation has been well characterized and shown to generate a complex, coordinated pattern of respiratory activity (Smith et al., 1990). Recordings of diaphragmatic EMG, cervical ventral roots, and hypoglossal roots provide information regarding inspiratory drive transmission to key components of the respiratory motor system. The respiratory motor discharge produced by wild-type mice preparations at E18.5 were regular and at a frequency similar to newborn pups. In marked contrast, the motor patterns generated by the preparations from *Ndn*^{tm2Stw} mice were very irregular, with prominent bouts of depression of respiratory rhythmogenesis that would account for the hypoventilation observed in newborn *Ndn*^{tm2Stw} mice *in vivo*. The abnormal respiratory discharge pattern was present at the level of the diaphragm, cervical ventral roots, cranial motoneuron pools and within neurons located in the putative respiratory rhythm-generating center, the pre-Bötzinger complex.

These data indicate that the defect in *Ndn*^{tm2Stw} mutant mice can be explained by abnormal respiratory rhythmogenesis emanating from the medulla. Data from *in vitro* (Smith et al., 1991) and *in vivo* (Ramirez et al., 1998; Solomon et al., 1999; Gray et al.,

Table 1. Characterization of inspiratory bursts in wild-type and mutant embryonic mouse preparations

	n	Interval(s)	Duration(s)	Amplitude (mV)	Coefficient of variation of burst interval
Wild-type en bloc	5	3.2 ± 2.3	0.32 ± 0.09	46 ± 21	0.72
Mutant en bloc					
Low frequency	7	32 ± 41*	0.33 ± 0.07	51 ± 34	1.3*
Medium frequency	7	3.3 ± 2.4	0.28 ± 0.05	37 ± 26	0.73
Combined average	7	8.5 ± 17.3*	0.28 ± 0.06	38 ± 27	2.0*
Wild-type slice	5	3.5 ± 2.8	0.30 ± 0.08	33 ± 18	0.8
Mutant slice					
Low frequency	8	24 ± 37*	0.29 ± 0.08	37 ± 21	1.5*
Medium frequency	8	3.8 ± 2.8	0.26 ± 0.06	27 ± 20	0.74
Combined average	8	7.1 ± 15.6*	0.27 ± 0.08	29 ± 20	2.2*

The mean interburst interval, duration, and amplitude of inspiratory bursts were calculated from recordings of inspiratory motor discharge generated by brainstem–spinal cord (en bloc) and medullary slice preparations from E18.5 mice. The measurements for mutant mice were calculated separately for bouts of low- and medium-frequency bursting and put together as a combined average. Results are means ± SD; *n* is the number of preparations examined. *$p < 0.05$ compared with wild type; Student's *t* test.

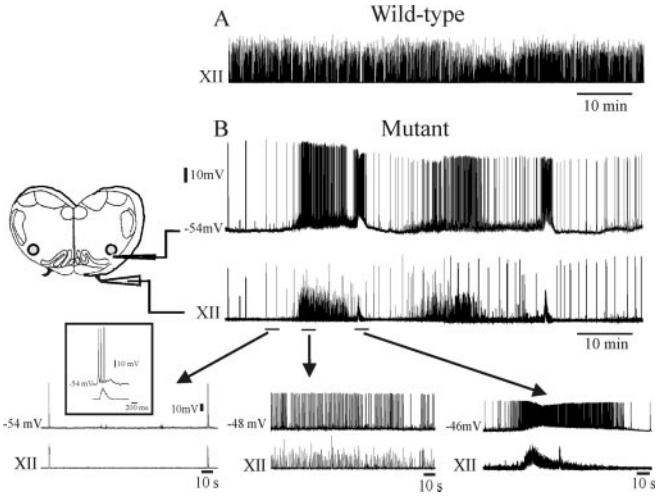

Figure 2. Abnormal rhythmogenesis is apparent from whole-cell patch-clamp recordings from an inspiratory neuron within the pre-Bötzinger complex. *A*, Rectified and integrated suction electrode recordings were made from the XII nerve roots of a wild-type E18.5 medullary slice preparation. *B*, *Top* shows whole-cell patch-clamp recording from an inspiratory neuron located within the region of the pre-Bötzinger complex. *Middle* shows the simultaneous recording from the XII nerve root. *Bottom* shows the whole-cell and nerve root recordings on a shorter time scale. The *traces* were taken from the areas demarcated in the *middle panel* with *horizontal bars*. The rhythmic discharge fluctuates between periods of very slow rhythms (*left bottom*) to those in which the respiratory rhythm is similar in frequency to wild-type preparations (*middle bottom*). There are also occurrences of high-frequency nonrespiratory bursts (*right bottom*). *Inset* shows whole-cell and integrated nerve recordings during a single inspiratory burst.

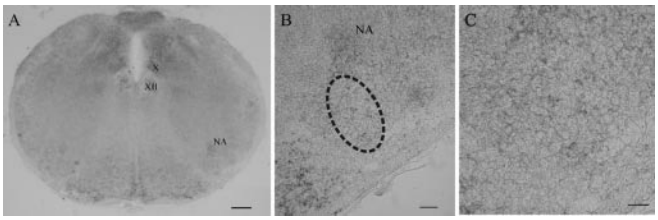

Figure 3. *necdin* is expressed in the fetal medulla. *A*, Expression of *Ndn* in E18.5 medullary transverse section equivalent to those used for electrophysiological studies. *B*, Photo of labeling in the ventrolateral medulla (pre-Bötzinger complex area approximated by *dashed line*). *C*, Higher-power photo of the pre-Bötzinger complex region. *NA*, Nucleus ambiguous; *X*, nucleus of the tenth nerve (vagus); *XII*, nucleus of the twelfth nerve (hypoglossal). Scale bars: *A*, 200 μm; *B*, 50 μm; *C*, 25 μm.

2001) models strongly suggest that a well defined region of the ventrolateral medulla, the pre-Bötzinger complex, is a major contributor to the genesis of respiratory rhythm. A detailed understanding of the cellular mechanisms underlying rhythm and pattern generation with the ventrolateral medulla remains to be elucidated. However, there are data to support a pacemaker-network hypothesis, which states that the kernel for rhythm generation consists of a population of neurons with intrinsic pacemaker properties that are embedded within, and modulated by, a neuronal network (Rekling and Feldman, 1998; Smith et al., 2000). It has been postulated that the pacemaker properties arise from intrinsic voltage-dependent conductances that confer increases in burst frequency at depolarized membrane potentials and decreases, to the point of inhibiting rhythmic bursting, at hyperpolarized membrane potentials (Smith et al., 1991; Butera et al., 1999a,b). The primary conditioning excitatory drive that maintains the oscillatory state arises from activation of glutaminergic receptors (Greer et al., 1991; Funk et al., 1993). Additional conditioning is provided by a diverse group of neuromodulators, including GABA, serotonin, noradrenaline, opioids, prostaglandins, substance P, and acetylcholine (Lagercrantz, 1987; Moss and Inman, 1989; Ballanyi et al., 1999). Thus, absence of *necdin* expression could result in the loss, or perturbation of function, of rhythmogenic neurons in the pre-Bötzinger complex. This is the proposed abnormality in *Rnx*-deficient mice, which also have a central respiratory defect, possibly attributable to altered cell-fate commitment of respiratory neurons attributable to loss of this homeobox transcription factor (Shirasawa et al., 2000; Qian et al., 2001). Alternatively, *necdin* expression may be necessary for the proper functioning of neurons providing appropriate conditioning drive impinging on rhythmogenic neurons within the pre-Bötzinger complex.

People with PWS are deficient for multiple genes, including *necdin*. Although many aspects of PWS can be related to a basic defect in hypothalamic development, development of other systems is probably also compromised in PWS. Abnormal ventilatory responses to hyperoxia, hypoxia, and hypercapnia when awake and sleeping are noted in PWS patients (Arens et al., 1994; Gozal et al., 1994; Schluter et al., 1997; Menendez, 1999). Furthermore, there are reports of sleep-related central and obstructive apnea (Clift et al., 1994; Wharton and Loechner, 1996; Manni et al., 2001; Nixon and Brouillette, 2002). A report of a 29 week premature infant with PWS who required prolonged ventilatory support points to a prenatal onset of respiratory dysfunction in PWS (MacDonald and Camp, 2001). The sleep-related breathing problems likely contribute significantly to the excessive daytime

sleepiness in childhood and adulthood that is characteristic of PWS (Hertz et al., 1995). Aside from one report showing reduced number of oxytocin neurons in the hypothalamic paraventricular nucleus, no abnormal pathological findings have been noted in PWS individuals at autopsy (Swaab et al., 1995). Our study now suggests that loss of *necdin* is implicated in abnormal respiration in PWS infants, and we hypothesize that *necdin* may be important for normal respiratory activity in the human newborn medulla.

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