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Prolactin Replacement Must Be Continuous and Initiated Prior to 21 d of Age to Maintain Hypothalamic Dopaminergic Neurons in Hypopituitary Mice

Carol J. Phelps,[1,2] Mario I. Romero,[1] and David L. Hurley[1,3]

[1]Neuroscience Program, and Departments of [2]Structural and Cellular Biology, and [3]Cell and Molecular Biology, Tulane University, New Orleans, LA

The prolactin (PRL) deficit in mice homozygous for the spontaneous Ames dwarf (df) mutation coincides with a marked reduction in the number of PRL-regulating tuberoinfundibular dopaminergic (TIDA) neurons. The TIDA deficit develops after 14–21 d postnatally and may be prevented by PRL replacement initiated at 12, but not at 60, d of age. The present study was designed to define further the developmental period during which PRL can prevent the deficit in the number of TIDA neurons in df/df mice, as well as to evaluate whether exposure to PRL neonatally affects the response to PRL by TIDA neurons in later development. To address the first aim, litters of df/df and normal (DF/df) mice were treated daily with ovine PRL (50 µg intraperitoneally), starting at 12, 21, or 30 d of age. To address the second aim, DF/df and df/df animals treated with PRL for 30 d starting at 12 d of age were subjected to PRL withdrawal (15 d of saline vehicle treatment), followed by PRL retreatment. All brains were evaluated using both catecholamine histofluorescence and tyrosine hydroxylase (TH) immunocytochemistry. Total numbers of TH-immunostained cells were counted in area A12 (TIDA neurons) and in A13 (medial zona incerta). Qualitatively, catecholamine fluorescence in A12 perikarya and terminals in df/df mice was enhanced by PRL treatment initiated at 12 or 21, but not at 30, d of age. TH immunostaining intensity was enhanced in all df/df PRL-treated groups, compared with saline treatment. However, total numbers of TH-positive TIDA neurons were reduced significantly in df/df mice treated with PRL beginning at 21 or 30 d, as well as with saline at 12 d, compared with similarly treated DF/df groups and with df/df animals treated with PRL beginning at 12 d ($p < 0.01$ for all comparisons). Among dwarf mice treated with PRL beginning at 12 d, followed by PRL withdrawal, the numbers of TH-positive TIDA neurons were greater than those of saline-treated dwarfs, but less than those in DF/df mice ($p < 0.05$ for both comparisons). In dwarfs retreated with PRL after withdrawal, the TIDA population was also smaller than that in normal animals ($p < 0.05$), although it was larger than in vehicle-treated dwarfs of the same age ($p < 0.05$). No effect of PRL treatment on TIDA cell numbers in normal mice, or of treatment or mouse phenotype on the number of TH-positive cells in zona incerta, occurred in either experiment. These results indicate that the effect of PRL on preventing the reduction in the TIDA population in df/df mice is limited to a developmental period prior to 21 d postnatally. In addition, this study provides evidence that continuous PRL feedback is required to maintain normal numbers of TIDA neurons. These findings extend the evidence for a critical role of PRL feedback in the differentiation and preservation of phenotype in TIDA neurons.

Key Words: Prolactin; hypothalamus; dopamine; arcuate nucleus; dwarf mouse; tyrosine hydroxylase.

Introduction

Prolactin (PRL) secretion is tonically inhibited by hypothalamic tuberoinfundibular dopaminergic (TIDA) neurons *(1,2)*, also known as catecholaminergic area A12 *(3)*. The axon terminals of TIDA neurons are known to project to the external zone of the median eminence (ME) *(4,5)*, where dopamine (DA) is released into the hypophysial portal circulation *(6)*. In turn, PRL regulates its own secretion via a short-loop feedback mechanism *(7)*, as demonstrated by increased ME DA synthesis *(8)*, turnover *(9)*, and release *(10,11)*, resulting in increased DA levels in portal plasma *(6)*, after PRL treatment.

Studies on the TIDA neurons in genetically PRL-deficient animals, such as Ames (df/df; *[12]*), Snell (dw/dw; *[13]*), and Snell-Jackson (dw^J/dw^J; *[14]*) dwarf mice, have suggested a role for PRL in the development of the TIDA neurons *(15)*. All these dwarf mouse types bear recessive

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Author to whom all correspondence and reprint requests should be addressed: Dr. Carol J. Phelps, Department of Structural and Cellular Biology, SL-49, Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, LA 70112. E-mail: cjphelps@tulane.edu

mutations that in the homozygous condition, result in failure of the anterior pituitary to initiate growth hormone and PRL synthesis *(16)*. Coincident with the absence of PRL in dwarf mice, a deficit exists in the expression of DA *(17,18)* and its synthetic enzyme, tyrosine hydroxylase (TH) *(19, 20)*, which is isolated to the TIDA neurons. Compared with normal mice, the df/df TIDA neuronal population is reduced to <50%, as assessed by TH immunocytochemistry *(20)*. Developmental studies showed that the TIDA neuron DA/TH deficit in Ames dwarfs has a postnatal onset, in that DA levels fail to increase *(21)*, and the number of TH-immunoreactive TIDA neurons decreases *(22)*, after 14 and 21 d of age, respectively.

In dwarf mice, continuous PRL replacement initiated at 12 d of age (i.e., prior to the occurrence of the DA deficit in the TIDA population) supported DA expression and a number of TH-immunoreactive A12 neurons comparable with that of normal littermate mice *(23)*. However, PRL treatment initiated at 60 d of age did not restore the TIDA population in df/df mice, although the treatment increased the intensity of TH immunoreactivity and catecholamine (CA) histofluorescence in extant TIDA neurons *(24)*.

To define better the critical developmental period during which PRL can prevent the decline in the number of df/df TIDA neurons, the effect of PRL replacement initiated at 21 and 30 d of age was compared with that of PRL treatment initiated at 12 d. This study also was designed to test whether transient exposure to PRL neonatally affects whether TIDA neurons can respond to PRL later in life. To address that question, dwarf mice that received PRL replacement initiated at 12 d of age were subjected to a period of PRL withdrawal followed by PRL retreatment. The experiment thus tested whether TIDA neurons are present and able to respond to PRL in adult dwarfs that received PRL during the critical period, as opposed to those that lacked PRL feedback throughout postnatal development. The effect of PRL on the TIDA neurons was evaluated by CA histofluorescence and TH immunocytochemistry (ICC).

Results

PRL Replacement at 21 and 30 d of Age

Catecholamine Histofluorescence

Figure 1 illustrates CA histofluorescence in TIDA perikarya and ME in representative midcoronal sections of DF/df (Fig. 1A–D) and df/df (Fig. 1E–H) mice treated with saline (Fig. 1A,E) or with PRL beginning at 12 (Fig. 1B,F), 21 (Fig. 1C,G), or 30 (Fig. 1D,H) d of age. In DF/df mice, CA histofluorescence was observed consistently in both perikarya and terminals, with some variability in intensity that appeared to be independent of the treatment (Fig. 1A–D); for example, perikarya in Fig. 1C are relatively dim, compared with those in Fig. 1A, 1B, or 1D, but ME fluorescence is intense in Fig. 1C. As reported previously *(23)*, CA histofluorescence in saline-treated dwarfs (Fig. 1E) was reduced markedly compared with saline-treated normal mice (Fig. 1A), in both cell bodies and ME. In df/df mice in which PRL treatment was initiated at 12 d of age (Fig. 1F), perikaryal CA histofluorescence intensity was qualitatively comparable with that of vehicle-treated DF/df mice (Fig. 1A) and was enhanced in both cell bodies and ME, compared to that of saline-treated dwarfs (Fig. 1E). In dwarf mice treated with PRL beginning at 21 d postnatally (Fig. 1G), CA histofluorescence was visible in A12 perikarya and ME but was less intense than in df/df mice treated with PRL starting at 12 d of age (Fig. 1F). By contrast, in df/df mice that receive PRL treatment beginning at 30 d postnatally (Fig. 1H), CA histofluorescence in TIDA cells and ME terminals was minimal and comparable with that of saline-treated dwarfs (Fig. 1E).

Tyrosine Hydroxylase ICC

Representative sections of TH immunostaining at midcoronal A12 and ME levels are illustrated in Fig. 2 for DF/df (Fig. 2A–D) and df/df (Fig. 2E–H) mice treated with saline (Fig. 2A,E) or with PRL beginning at 12 (Fig.2B,F), 21 (Fig. 2C,G), or 30 (Fig. 2D,H) d of age. Intensity of TH immunostaining was qualitatively similar in the TIDA cell bodies of DF/df mice, regardless of the treatment (Fig. 2A–D). In the ME, intensity of TH immunoreactivity in DF/df mice appeared to be greater after PRL (Fig. 2B–D) than saline (Fig. 2A) treatment. Saline-treated dwarf mice (Fig. 2E) showed reduced TH immunoreactivity in both TIDA perikarya and ME compared with vehicle-treated normal mice (Fig. 2A). Immunostaining for TH in P12 dwarf mice (Fig. 2F) was increased markedly in both perikarya and ME compared with that of saline-treated df/df mice (Fig. 2E) and was comparable with that observed in DF/df animals (Fig. 2A–D). In both P21 (Fig. 2G) and P30 (Fig. 2H) df/df mice, TH immunoreactivity was enhanced qualitatively, especially in perikarya, compared with saline-treated dwarfs (Fig. 2E).

Total TH-Positive Cell Numbers

Total numbers of TH-immunoreactive cells are shown in Fig. 3 for areas A12 (Fig. 3A) and A13 (Fig. 3B). In A12, two-factor analysis of variance (ANOVA) revealed that the total TH-immunoreactive cell number was different for phenotypes ($F_{1,49} = 78.11$; $p = 0.0001$), and that treatment ($F_{3,49} = 3.93$; $p < 0.05$) and interaction of type and treatment ($F_{3,49} = 5.09$; $p < 0.01$) had significant effects on the A12 TH-immunoreactive population. In DF/df mice, TH-positive TIDA neurons ranged from 2861 ± 196 (P21) to 3366 ± 274 (S12) and averaged 3129 ± 124 cells; there was no effect of treatment among DF/df groups.

The TH-positive TIDA population in dwarf mice numbered 1237 ± 157 in S12, 2607 ± 264 in P12, 1785 ± 76 in P21, and 1604 ± 75 in P30 groups. One-factor ANOVA (assessing type and treatment together as independent variables) revealed that the TH-positive TIDA population was reduced significantly in S12, P21, and P30 df/df mice com-

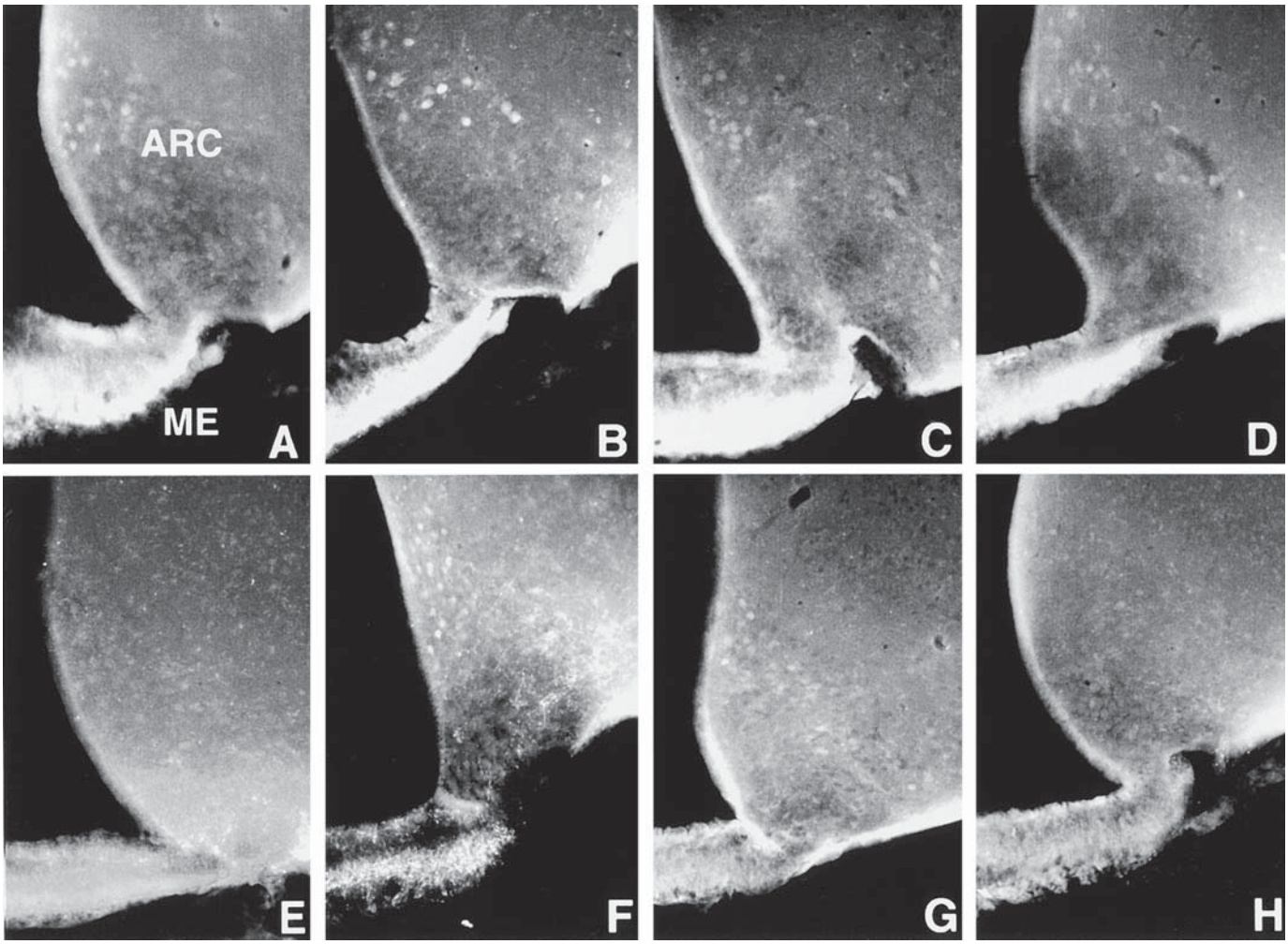

Fig. 1. Catecholamine histofluorescence in TIDA perikarya in arcuate nucleus (ARC) and ME terminals in normal (**A–D**) and dwarf (**E–H**) mice treated with saline (A,E) or with PRL beginning 12 (B, F), 21 (C,G), or 30 (D,H) d of age. Coronal 30-µm sections; original objective magnifications: ×20.

pared with similarly treated DF/df mice, and with P12 df/df animals ($p < 0.01$ for all comparisons). The number of A12 TH-positive cells did not differ between P21 and P30 df/df mice. No significant differences in the number of TH-positive cells in A13 were observed regarding treatment or phenotype (Fig. 3B).

Effect of PRL Treatment Withdrawal, and Retreatment

PRL treatment was initiated at 12 d in both DF/df and df/df mice. After 30 d of treatment, at age 42 d, one group of mice was injected daily with saline vehicle for 15 d (PS), and another group was retreated with PRL (30 d) after 15 d of saline (PSP). Control normal and dwarf mice were treated continuously with saline vehicle (SS, SSS).

Catecholamine Histofluorescence

Figure 4 illustrates CA histofluorescence in A12 perikarya and ME in representative midcoronal sections of DF/df (Fig. 4A,B) and df/df (Fig. 4C,D) animals treated with either PRL followed by saline vehicle (PS; Fig. 4A,C) or PRL retreatment after PS (PSP; Fig. 4B,D). The maintenance of CA histofluorescence in the TIDA neurons in df/df mice treated continuously with PRL from 12 d of age (Figs. 1F and 4D) was absent in dwarfs subjected to PRL withdrawal (Fig. 4C); fluorescence in PS df/df mice was comparable with that in saline-treated df/df mice (Fig. 1E). CA histofluorescence in dwarf mice treated with PRL subsequent to the PRL withdrawal period (PSP; Fig. 4D) was enhanced compared with that in vehicle-treated (Fig. 1E) and PS (Fig. 4C) df/df mice, and it was comparable with that in DF/df animals (Fig. 4A,B), although cell number appeared to be reduced compared with PSP normal animals. The CA histofluorescence in TIDA perikarya and ME of DF/df mice also appeared to be enhanced by PRL treatment (Fig. 4B vs 4A).

Tyrosine Hydroxylase ICC

Figure 5 shows representative TH immunostaining in midcoronal sections of A12 perikarya and ME in DF/df (Fig. 5A,B)

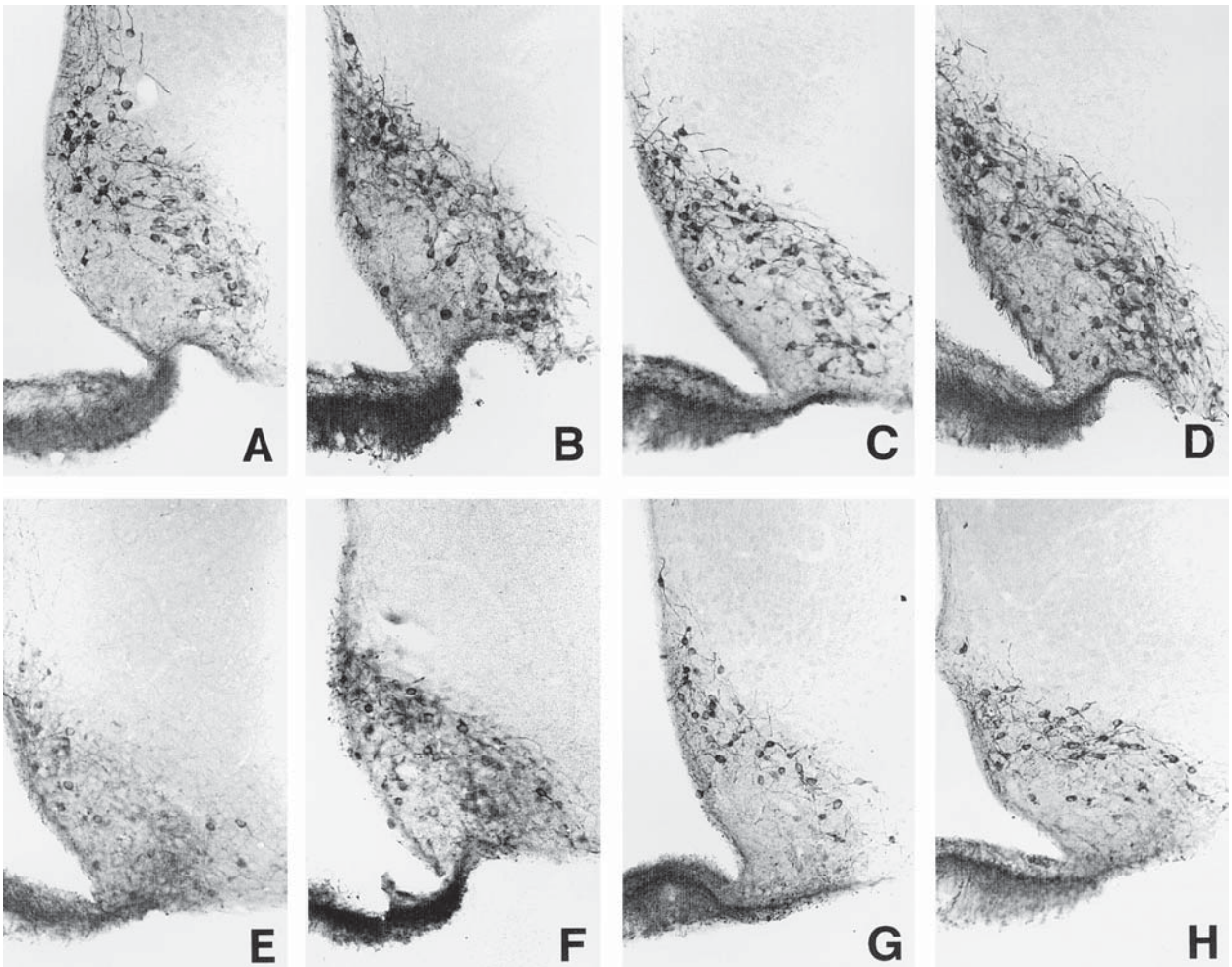

Fig. 2. TH immunoreactivity in TIDA perikarya and ME terminals in normal (**A–D**) and dwarf (**E–H**) mice treated with saline (A,E) or with PRL beginning at 12 (B,F), 21 (C,G), or 30 (D,H) d of age. Coronal 30-μm sections; original objective magnifications: ×20; bar in (A) represents 100 μm.

and df/df (Fig. 5C,D) animals treated with either PRL followed by saline vehicle (PS; Fig. 5A,C) or PRL retreatment after PS (PSP; Fig. 5B,D). TH immunostaining intensity was enhanced in both cell bodies and ME in normal (PSP) mice (Fig. 5B) compared with normal mice subjected to PRL withdrawal (PS; Fig. 5A). Dwarf mice treated with vehicle for 2 wk after PRL begun at 12 d of age (PS) showed TH immunostaining (Fig. 5C) that was comparable with that of dwarfs treated with saline alone (Fig. 2E). Resumed PRL treatment in dwarfs (PSP) resulted in TIDA neuron and ME TH immunoreactivity that was qualitatively comparable with that in DF/df mice, and enhanced compared with saline-treated (Fig. 2E) and PS (Fig. 5C) df/df animals.

Total TH-Positive Cell Numbers

Numbers of TH-immunoreactive cells in DF/df and in df/df mice subjected to PRL withdrawal (PS) and retreatment (PSP) are graphed in Fig. 6 for TIDA neurons (A12) (Fig. 6A), and medial zona incerta (A13) (Fig. 6B). In A12, significant effects of type ($F_{1,43} = 94.22$; $p = 0.0001$) and type-treatment interaction ($F_{3,43} = 5.09$; $p < 0.01$), but not of the treatment, were revealed by two-factor ANOVA. The total number of TH-positive TIDA neurons in DF/df mice did not differ among treatment groups and together averaged 3525 ± 110 neurons. In saline-treated dwarfs, TH-immunoreactive TIDA neurons totaled 1793 ± 101 at 57 d of age (SS) and 1685 ± 157 at 87 d of age (SSS). In both saline-treated df/df groups, the number of total TH-positive cells was reduced significantly compared with DF/df animals of the same age ($p < 0.01$ for both comparisons). Numbers of A12 TH-positive neurons were similar for PS (2408 ± 190) and PSP (2438 ± 232) df/df mice. In both groups (df/df PS and PSP), the TH population was reduced compared with that in similarly treated DF/df mice ($p < 0.05$ for both comparisons) but was increased compared with that of saline-treated dwarfs of the same age ($p < 0.01$ for both compar-

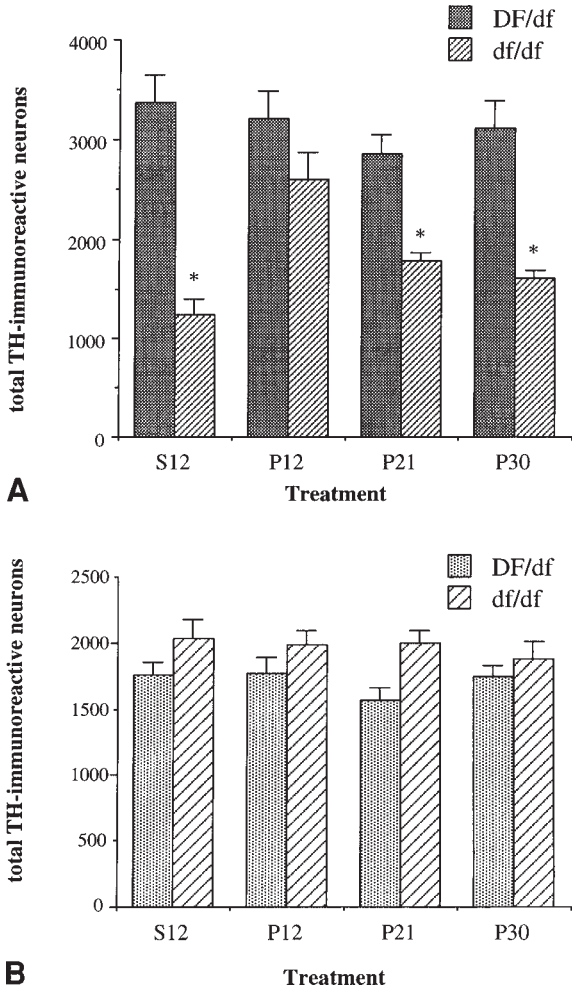

Fig. 3. Total TH-immunoreactive cells in (**A**) A12 (TIDA neurons) and (**B**) A13 (medial zona incerta). Columns represent mean, and vertical bars represent SEM. DF/df = normal, df/df = dwarf; P = PRL; S = saline vehicle; 12, 21, and 30 = d of age at which the treatment was initiated. *$p < 0.01$, compared with similarly treated DF/df mice.

isons). In addition, A12 TH-positive cell numbers in both df/df PS and PSP groups were higher than in P30 df/df (Fig. 3A; $p < 0.05$), and in PS-treated dwarfs were higher than in the P21 df/df mice (Fig. 3A; $p < 0.05$). There were no significant differences among vehicle-treated df/df groups; that is, A12 cell number was comparable for dwarf S12 (Fig. 3A), SS, and SSS. The number of TH-positive cells in A13 was similar in all treatment groups and both phenotypes (Fig. 6B).

Discussion

The results of these studies indicate that a critical postnatal period exists for the initiation of PRL feedback to support the continued development of TIDA neurons. Previous experiments showed that PRL replacement initiated at 12 d of age in genetically PRL-deficient Ames dwarf mice resulted in a complement of TH-immunoreactive TIDA neurons that was equal to that in normal mice *(23)*, thus sustaining a population that is otherwise deficient in dwarfs *(20)* because of a postnatal decline in number *(22)*. Treatment of adult (60 d-old) Ames dwarfs with PRL was without effect on the number of TIDA neurons *(24)*, indicating that the stimulatory effect of PRL is required prior to 60 d of age. The present findings show that PRL feedback is required prior to 21 d of age to support the development of a normal TIDA population, narrowing the identified critical period to between 12 and 21 d of age. Dwarfs treated with exogenous PRL beginning at 21 or 30 d of age showed numbers of TH-immunoreactive TIDA neurons that were significantly lower than those in similarly treated or untreated normal littermates and not different from numbers in untreated dwarfs.

That a stimulatory effect of PRL is necessary for normal development of TIDA neurons, and that there is an early critical period for initiation of this effect, are corroborated by studies in neonatal rats *(25)*. Reduction in PRL levels in milk by treating lactating rats with bromocriptine on d 2–5 postpartum resulted in reduced DA levels and turnover in the ME of offspring *(26)*. Rats subjected to this deficiency in PRL neonatally also exhibited hyperprolactinemia that persisted at 100 d of age *(27)*, indicating defective activity of TIDA neurons. Delaying the bromocriptine-induced PRL deficiency to 9–12 d postpartum did not disrupt TIDA neuron activity in the offspring, suggesting a narrow neonatal critical period for PRL stimulation of TIDA neuron development. Note that the developmental pattern of pituitary PRL translation is different for rats and mice, such that PRL production and secretion begins at 3 to 4 d of age in rats *(28)*, while PRL is not detectable in mouse pituitary until 7 to 8 d postnatally *(21,29)*. The critical period for initiation of PRL effect is therefore likely to occur earlier in the development of rats, and this difference in timing is supported by the collective previous and current studies.

CA histofluorescence in A12 was enhanced in dwarfs treated with PRL beginning at 21 d and intensity of TH immunostaining appeared to be greater in dwarfs treated with PRL at either 21 or 30 d, compared with vehicle-treated dwarfs. Enhanced intensity of TH immunostaining in extant TIDA neurons in adult dwarfs treated with PRL was quantified using relative optical density measurements in a previous study *(24)*, and the qualitative assessment in the present study is likely to represent similarly enhanced TIDA neuron activity in response to acute PRL stimulation as documented in rats *(30)*.

Because a previous developmental study *(22)* documented that TIDA neuron number is not significantly lower in Ames dwarf than in normal littermates at 21 d of age, it was surprising that PRL replacement beginning at that age did not maintain a TIDA neuronal population comparable with that in normal mice, or at a greater size than in dwarfs treated with PRL beginning at 30 d. The data suggest that the mechanism(s) responsible for the decline in numbers of detectable TH-immunoreactive TIDA neurons in PRL-deficient

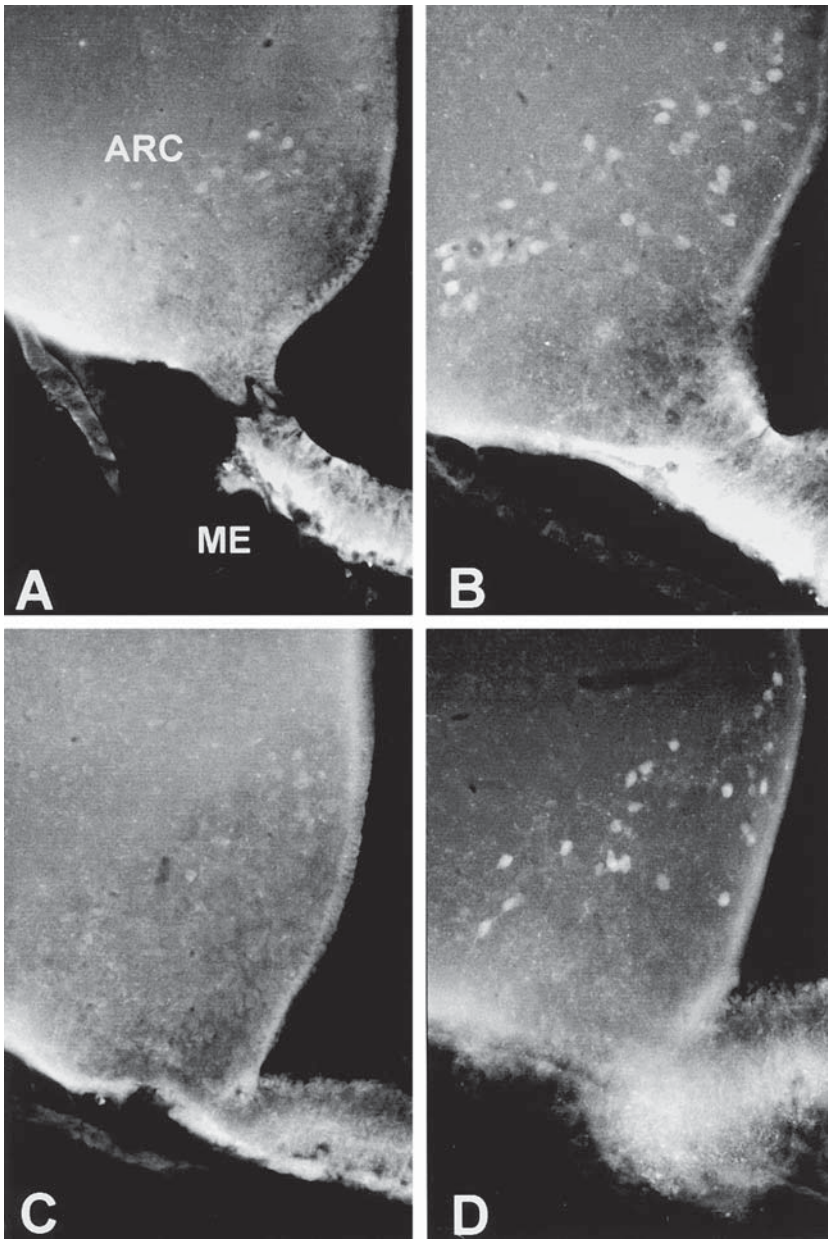

Fig. 4. CA histofluorescence in area A12 and ME in representative midcoronal levels in DF/df (**A,B**) and df/df (**C,D**) animals treated beginning at 12 d of age with either PRL for 30 d, followed by saline vehicle for 15 d (A,C), or with PRL for 30 d after the previous PRL-vehicle regimen (B,D). Coronal 30-µm sections; original objective magnifications: ×20.

dwarf mice are irreversible by 21 d postnatally. Identification of these mechanisms and definition of their developmental pattern will be required to explain the time course of decline and responsiveness to PRL.

Adult Ames dwarfs subjected to lifelong absence of PRL feedback were unresponsive to exogenous PRL in terms of restoring TH-immunoreactive TIDA neuron number *(24)*. Therefore, it was important to investigate whether adult dwarfs exposed to PRL neonatally would later exhibit responsiveness to PRL in those terms. The results of PRL treatment, withdrawal, and retreatment suggest that exposure to PRL beginning at 12 d of age, and continuing through 42 d, is not adequate if discontinued to maintain the capability of producing detectable amounts of TH by a portion of the TIDA neurons. Cessation of PRL feedback, effected by withdrawal of PRL replacement in 42 d-old dwarfs, resulted in a TIDA population that was significantly smaller that that in normal mice, including failure of subsequent PRL treatment to restore the TIDA neuronal population.

This effect can be compared with that of hypophysectomy in adult rats. Adult hypophysectomy and subsequent hormone replacement tests hypophysiotropic neuronal response in animals exposed to important developmental feedback effects by pituitary hormones. Removal of the pituitary

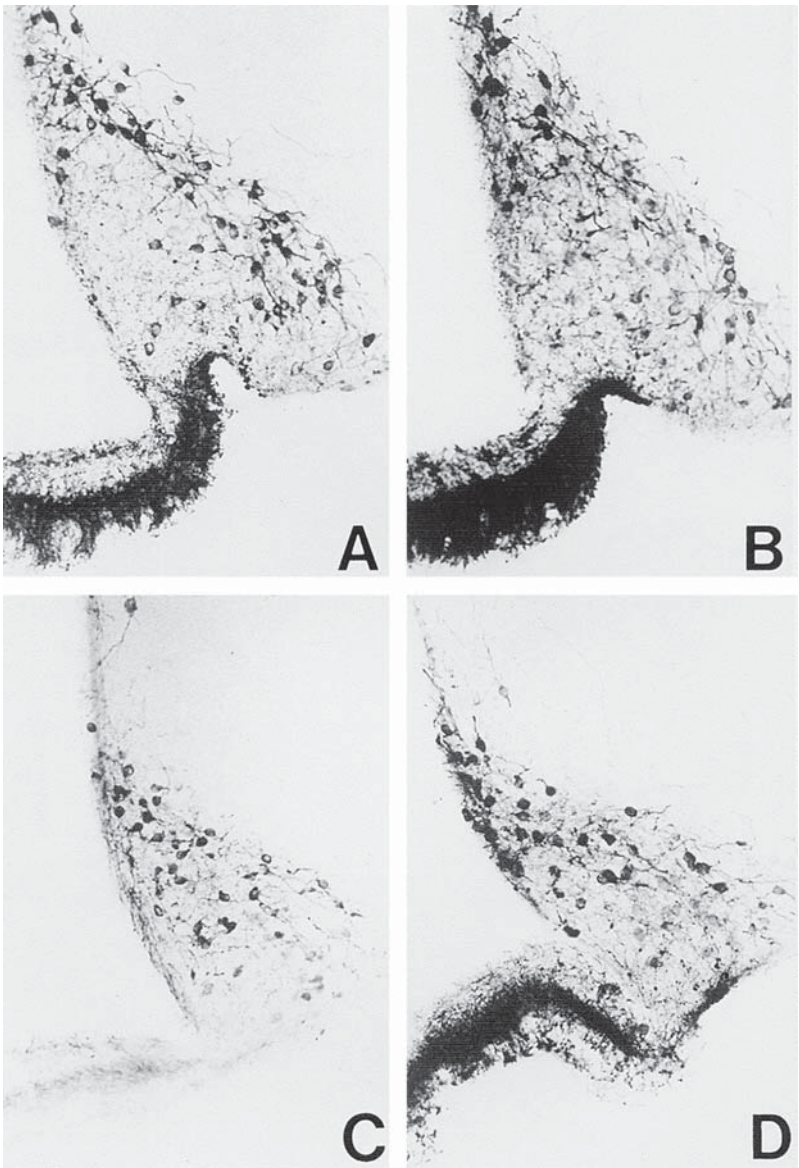

Fig. 5. TH immunocytochemical staining in area A12 and ME in representative midcoronal levels in DF/df (**A,B**) and df/df (**C,D**) animals treated beginning at 12 d of age with either PRL for 30 d, followed by saline vehicle for 15 d (A,C), or with PRL for 30 d after the previous PRL-vehicle regimen (B,D). Coronal 30-μm sections; original objective magnifications: ×20.

and subsequent PRL replacement has resulted in decreased TIDA neuron responsiveness to PRL short-loop feedback effects in terms of ME DA turnover *(31)*. However, numbers of detectable TH-producing TIDA neurons or total TH protein levels in hypothalamus have not been assessed after adult hypophysectomy. In addition, hypophysectomy rarely is performed in rats as young at 42 d, the age at which PRL withdrawal was begun in Ames dwarfs in the current study. It would be interesting to determine whether TIDA neuron number is reduced after hypophysectomy, and restored after PRL treatment, in adult rats or mice; such experiments have not been reported.

In the present study, TIDA neuron number in dwarfs exposed to PRL between 12 and 42 d and then subjected to withdrawal or retreatment was enhanced compared with vehicle-treated dwarfs, although lower than in normal mice. In dwarfs retreated with PRL after a 2-wk withdrawal period, both CA histofluorescence and intensity of TH immunoreactivity in extant A12 neurons were enhanced compared with dwarfs evaluated after PRL treatment withdrawal or after continuous treatment with vehicle, similar to findings in adult Ames dwarfs treated with PRL: extant TIDA neurons are stimulated by PRL. The data on the partial effect of both PRL treatment begun at 12 d and then withdrawn, and of

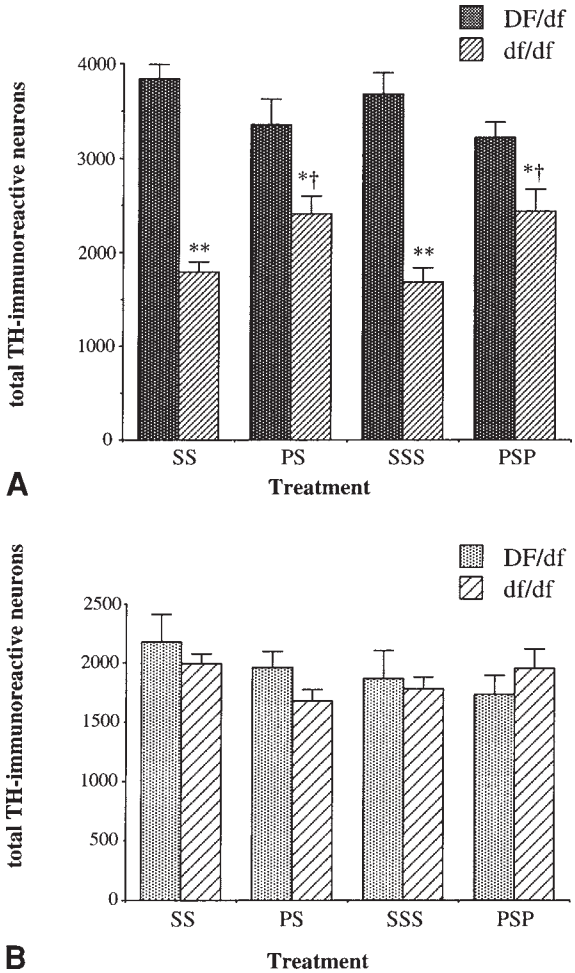

Fig. 6. Total TH-immunoreactive cells in (**A**) A12 and (**B**) A13 in normal (DF/df) and dwarf (df/df) mice treated with alternating sequence of PRL (P) for 30 d and saline vehicle (S) for 15 d, beginning at 12 d of age. SS and PS groups were 57 d old; SSS and PSP groups were 87 d old. Columns represent means, and vertical bars represent SEM. $*p < 0.05$, $**p < 0.01$, compared with similarly treated DF/df mice; $^{\dagger}p < 0.05$, compared with saline-treated df/df mice of the same age.

PRL retreatment after withdrawal in genetically PRL-deficient mice support both the existence of a critical postnatal period for an effect of PRL on development of TIDA neurons and the requirement of continuous PRL feedback to maintain phenotype in a normal complement of these cells.

The mechanism of PRL effect on TIDA neurons, as well as the access of PRL to target sites in the central nervous system (CNS), has not been clearly established. Direct stimulation of TIDA neurons by PRL would, at least, require PRL receptors (PRLRs) on those neurons. Expression of PRLR mRNA *(32–34)* and protein *(35)* in the medial basal hypothalamus has been reported, as has colocalization of PRLR with TH in TIDA neurons in vitro *(36)* and in adult brain sections *(37)*. Intracerebroventricular administration of PRL has resulted in c-Fos expression in some TIDA neurons *(38)*, and peripheral PRL injection has been shown to lead to nuclear Stat5 translocation and increased expression of Fos-related antigens in these neurons *(39)*, all suggesting activation of transcription. The localization of PRLR in mouse CNS, and the developmental pattern of PRLR expression in brain, have not been reported. Access of molecules as large as PRL to the CNS is problematic, considering the blood-brain barrier. However, studies in rats have indicated that the ventral hypothalamus is defective in preventing access of large molecules such as peroxidase *(40)*, suggesting that PRL in the general circulation might have direct access to the TIDA neurons.

In summary, the results of the present study indicate that PRL feedback stimulation is required for development of TIDA neurons, and that there is a critical developmental period between 12 and 21 d of age during which the PRL effect must be initiated. In addition, continuous PRL feedback appears to be required to maintain the entire TIDA population.

Materials and Methods

Animals

Dwarf (df/df) and normal (DF/df) mice were reared from matings of DF/df females with df/df males. Dwarf males were treated beginning at 6–8 wk of age with D/L-thyroxine (2 μg intraperitoneally three times per week; Sigma, St. Louis, MO), followed by pituitary renal capsule allografts from DF/df donors, as sources of homologous PRL to induce fertility. The pituitary transplant surgery was performed in anesthetized (100 mg/kg of ketamine, 10 mg/kg of acepromazine) animals. Normal pituitary donors were male or female, 2–6 mo old; they were anesthetized with ether and decapitated for pituitary gland removal. Whole single glands were placed under the kidney capsules of recipient animals. After surgery, recipients were observed continuously, and body temperature was supported, until recovery from anesthesia. The breeding colony was maintained under conditions of controlled temperature (22 ± 2ºC), with lights on from 6:00 AM to 6:00 PM, and food and water were available ad libitum. Procedures for maintenance, recovery surgery, and euthanasia were approved by the Tulane University School of Medicine Animal Care and Use Committee.

PRL Treatment

Entire litters (consisting of approximately half DF/df and half df/df mice) were treated daily with ovine PRL (50 μg intraperitoneally, oPRL-19; National Hormone and Pituitary Program [NHPP], National Institute of Diabetes and Digestive and Kidney Diseases [NIDDK]) starting at 12 (P12), 21 (P21), or 30 (P30), d of age; litters treated with daily injections of saline vehicle (0.03 *M* $NaHCO_3$ /0.15 *M* NaCl, pH 9.5) beginning at 12 d of age (S12) served as controls. These animals were euthanized at 42 d of age (1 d after the last injection).

Separate groups of animals were used to evaluate whether early PRL exposure affects the response of TIDA neurons to PRL feedback at older ages. Entire litters were treated, starting at 12 d of age, with PRL for 30 d, followed by saline for 15 d (PS); additional litters received the PS regimen, followed by retreatment with PRL for an additional 30 d (PSP); that is, PS mice were 57 d old, and PSP mice were 87 d old at evaluation. Animals used as controls for PS and PSP were treated with saline vehicle substituted for PRL, i.e., initiated at 12 d of age and continued until 57 (SS) or 87 (SSS) d of age.

Tissue Preparation and Induction of Catecholamine Histofluorescence

Deeply anesthetized (80 mg/kg of body wt pentobarbital) animals were perfused transcardially with 0.9% NaCl/0.5% $NaNO_3$, followed by buffered 4% paraformaldehyde/0.5% glutaraldehyde (Faglu) fixative *(41)*. After perfusion, brains were removed and postfixed for at least 24 h at 4ºC, then sectioned coronally at 30 µm using a Vibratome (Lancer, Technical Products, St. Louis, MO). Every sixth section (i.e., at every 180-µm interval) was mounted in rostral-to-caudal order and examined using narrow-band excitation wavelengths (405–410 nm) and a violet barrier filter (460 nm) on a Nikon Optiphot microscope equipped for fluorescence epiillumination. Remaining tissue sections were either processed for TH ICC immediately or stored in "cryoprotectant" solution *(42)* at –20ºC until processed.

Immunocytochemistry

To remove residual fixative and allow antibody access to glutaraldehyde-fixed linkages, brain sections were pretreated with 1% aqueous sodium borohydride. After extensive buffered phosphosaline rinsing, nonspecific staining was minimized by incubating sections with 1.5% normal goat serum for 1 h, prior to incubating with rabbit polyclonal anti-TH antiserum (1:5000; Chemicon, Temecula, CA) for 48 h. The tissue sections were processed further using biotinylated goat anti–rabbit IgG and avidin-biotin complex solutions (Vectastain kit; Vector, Burlingame, CA). Visualization of TH immunoreactivity was achieved by color development using 0.02% diaminobenzidene tetrahydrochloride and 0.003% H_2O_2 in Tris buffer. Sections then were mounted, dried, and cover-slipped using Permount (Fisher, Pittsburgh, PA) for microscopic examination. Sections from control and PRL-treated mice were immunostained simultaneously using identical antiserum aliquots and reagent solutions.

Quantification of TH-Positive Neurons

Cell counts were performed at 180-µm intervals through A12 (TIDA neurons) and A13 (medial zona incerta). Cell counts were corrected for sampling periodicity (X6, for every sixth section) to obtain total cell numbers. Because the sampling distance exceeded the average diameter of TIDA neurons (13.3 ± 1.3 µm) *(43)*, cell counts were not corrected for sampling error owing to missed or recounted cells *(44)*. The accuracy of the periodicity correction has been verified previously by counting cells in every section of separate animals, which closely approximated totals obtained by uniform sampling and correction *(20)*.

Statistical Analyses

Statistical evaluation of cell counts was accomplished by analysis of variance (ANOVA) (SuperANOVA program; Abacus Concepts, Berkeley, CA). Analysis of TH-positive cell counts grouped males and females together; previous analysis has shown that sex, either alone or interacting with age or phenotype, does not affect significantly the number of TIDA neurons in df/df mice *(20)*. Analysis of effects of genetic type, treatment, and type/treatment interaction was accomplished by two-factor ANOVA. Comparisons among the individual groups were made using one-factor ANOVA followed by Student-Newman-Keuls multiple range test. Differences <5% probability level were considered significant.

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