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Research report

Ontogeny of brain-derived neurotrophic factor gene expression in the forebrain of prairie and montane voles

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Abstract

Brain-derived neurotrophic factor (BDNF) plays an important role in normal brain development. In the present study, we examined the ontogenetic pattern of BDNF gene expression in both monogamous prairie voles (*Microtus ochrogaster*) and promiscuous montane voles (*M. montanus*); two closely related microtine rodents that differ in life strategy and social behavior. In both species, BDNF mRNA showed an early appearance and a transient expression in a regionally specific manner. In the dentate gyrus and CA3 region of the hippocampus, BDNF mRNA was found neonatally, increased gradually during development, and reached a peak at weaning, followed by a subsequent decline to the adult level. In the paraventricular nucleus of the hypothalamus, levels of BDNF mRNA persisted until weaning, followed by a significant increase to the adult levels at 3 months of age. BDNF mRNA also demonstrated a species-specific developmental pattern. In the cingulate cortex, BDNF mRNA labeling displayed a transient increase in the second and third postnatal weeks followed by a subsequent decrease to the adult level in prairie voles, but persisted throughout the course of development in montane voles. In general, montane voles achieved an adult pattern of BDNF mRNA expression earlier than did prairie voles. Together, these data indicate that BDNF may function differently in infant and adult brains, and that the two species of voles differ in the ontogenetic pattern of BDNF mRNA expression in a regional-specific manner, which may be associated with their different life strategy and brain and behavioral development.

Theme: Development and regeneration

Topic: Expression and regulation of trophic factors: neurotrophins

Keywords: Brain-derived neurotrophic factor; Neurotrophin; Development; Vole; *Microtus*; Social organization

1. Introduction

Voles are a group of microtine rodents that show remarkable diversities in life strategy and social behavior. For example, the prairie vole (*Microtus ochrogaster*) is a highly social monogamous species, in which both males and females display mating-induced pair bonding and biparental behavior. In contrast, the montane (*M. montanus*) and meadow voles (*M. pennsylvanicus*) are promiscuous species that are non-social with only females providing parental care [5,10,23]. Monogamous and promiscuous voles also differ in the rate of brain, body, and behavioral development [19,37,38], as well as in some neurotransmitter systems, such as vasopressin and oxytocin, which regulate social behavior [3,24,25,55,58]. Due to their close taxonomic relationship and profound differences in social organization and reproductive biology, voles provide an ideal model system for comparative studies [4,10,55,58]. In addition, voles (Microtinae) are related at the family level (different subfamily) to other common laboratory rodents such as rats and mice (Murinae) and hamsters (Cricetinae) [41], making their data valuable for more extended comparisons.

Recently, neurogenesis has been studied in the adult vole brain. The rate of cell proliferation in the dentate gyrus of the hippocampus or the subventricular zone was enhanced after female prairie voles were exposed to or mated with a male, and this increase in cell proliferation was attributed to an increased level of circulating estrogen associated with estrus induction [14,51]. In meadow voles,

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the rate of cell proliferation in the hippocampal formation showed a seasonal fluctuation, which was related to changes in the levels of adrenal and gonadal steroid hormones [16]. Although steroid hormones have been found to influence the expression of brain-derived neurotrophic factor (BDNF) [18,46,48–50], and BDNF regulates cell proliferation and survival in rats [31,59], at present little is known for the role that BDNF may play in the regulation of cells proliferation in voles.

BDNF is broadly distributed throughout the central nervous system (CNS) in a variety of species, including birds [1], rats [6,7,57], mice [21], non-human primates [20,27], and humans [8,39,44]. BDNF plays an important role in promoting proliferation, survival, growth, and differentiation for a broad range of neuronal cell types, including cholinergic neurons [2], dopaminergic neurons [22,32], serotonergic neurons [35], hippocampal neurons [17,40], striatal neurons [53], and motoneurons [56]. In regard to its critical importance for normal brain development, attempts have been made to study the ontogenetic pattern of the BDNF gene or its protein in the whole brain [12], the forebrain or brainstem [15,34], the cerebellum [45], and the retina [43]. Although these studies have provided some information regarding BDNF's effects during development, all experiments were performed exclusively in rats, and thus no information is available for the BDNF ontogeny in other animals. In the present study, we used in situ hybridization to examine the distribution pattern of BDNF mRNA in the vole brain during development. The purpose was 2-fold: to map regional BDNF mRNA expression at various stages during development and to compare the monogamous prairie voles versus the promiscuous montane voles on their ontogenetic patterns of BDNF mRNA expression.

2. Materials and methods

2.1. Subjects

Subjects were the F2 generation of laboratory-breeding colonies that started with field captured prairie voles (*M. ochrogaster*) and montane voles (*M. montanus*). Breeding pairs for each species were housed in plastic cages (20× 50×40 cm) that contained cedar chip bedding. Water and food were provided ad libitum. The cages were maintained on a 14:10-h light:dark photoperiod with lights on at 07:00 h, and the temperature was around 20±1°C. The colony was checked daily. Subjects from each species were decapitated either on the day of birth (Day 1), at 1 week, 2 weeks, or 3 weeks of age. In addition, some subjects were weaned at 3 weeks of age, housed in pairs in same-sex cages, and maintained under the same conditions as their parents until they were decapitated at 3 months of age. Following decapitation, each brain was removed immediately, frozen on dry ice, and subsequently cut on a cryostat. Brains were cut into 20-μm coronal sections, thaw-mounted on Superfrost/Plus slides (Fisher), and stored at −80°C until they were processed for BDNF in situ hybridization.

2.2. BDNF in situ hybridization

A 500-base pair rat BDNF cDNA was provided by Dr. Mark Smith (NIH). The cDNA was generated by RT-PCR from rat hippocampal mRNA, the antisense primer was 28-mer 5′-GGGTCGACAGTTGGCCTTTTGATACCGG-3′ and the sense primer was 28-mer 5′-GGTCTAGAT-CCACGGCCAAGGCAACTTG-3′ [50]. The [^{35}S]CTP-labeled BDNF antisense riboprobe was transcribed using T7 RNA polymerase from *Kpn*I-digested pGEM4Z-rBDNF, and the BDNF control sense probe was transcribed using Sp6 RNA polymerase from the same plasmid digested with *Hin*dIII. The probe was characterized previously [49].

BDNF in situ hybridization was performed according to the procedure described before [49]. Briefly, slide-mounted coronal sections at 100-μm intervals were fixed for 20 min in 4% paraformaldehyde in a phosphate buffer solution (PBS, pH 7.4), digested for 15 min with 5 μg/ml proteinase K in a TE buffer (50 mM Tris–HCl and 5 mM EDTA, pH 8.0), and then incubated for 10 min in 0.1 M triethanolamine (pH 8.0) containing 0.25% acetic anhydride. Slides were washed in 2× SSC, dehydrated in ascending ethanol, delipidated in chloroform, rinsed in 95% ethanol, and air-dried. Prehybridization was performed by applying to each slide 150 μl hybridization buffer containing 2.5 mg/ml tRNA at 55°C for 2 h. The hybridization buffer contained 62.5% formamide, 12.5% dextran sulfate, 37.5 mM NaCl, 10 mM Tris–EDTA (pH 8.0), 1.25× Dehardt's solution, and 10 mM DTT. After prehybridization, slides were washed with 2× SSC, dehydrated, air-dried, and then applied with 150 μl hybridization solution containing 0.5 mg/ml tRNA and 5×10^6 cpm/ml labeled probe. Slides were then covered with parafilm and hybridized at 37°C for 16 h. Thereafter, slides were washed in 50% formamide and 0.1% β-mercaptoethanol in 2× SSC for 2×15 min at 55°C, digested in 25 μg/ml RNaseA in RNase buffer (8 mM Tris–HCl, 400 mM NaCl, 0.8 mM EDTA, pH 8.0) for 30 min at 37°C, blocked in 1 mM DTT in the same buffer for 30 min at 37°C, washed again in 50% formamide and 0.1% β-mercaptoethanol in 2× SSC for 2×15 min at 55°C, and then washed in 50% formamide and 1% β-mercaptoethanol in 2× SSC for 2×30 min at 55°C. After dehydration, slides were air-dried and put on BioMax MR film (Kodak) along with [^{14}C] microscales (Amersham, Buckinghamshire, UK) for 2–3 days to generate autoradiograms. Afterwards, slides were dipped into NTB-2 track emulsion (Kodak; 1:1 with 0.6 M ammonium acetate, pH 3.5) and exposed for 3 weeks at 4°C. After developing, slides were stained with 0.5% Cresyl violet, dehydrated,

and cover-slipped. Control sections were hybridized with a ^{35}S-labeled sense probe, which did not generate any specific labeling.

2.3. Data analysis

The number of subjects for each age group per species ranged from six to 11. All slides were coded so that the experimenter was blind to the identity of the specimens. BDNF mRNA quantification was performed from the autoradiograms using a computerized image program (NIH IMAGE 1.60), permitting the conversion of optical density to nCi/tissue equivalents from the standard curve derived from coexposed standards. The background counting was subtracted from the total counting. The density of BDNF mRNA labeling was measured in the hippocampal formation (corresponding to Plates 30–32 in Paxinos and Watson [42]), amygdaloid complex (corresponding to Plates 31–33 in Paxinos and Watson [42]), cingulate cortex (corresponding to Plates 14–16 in Paxinos and Watson [42]), paraventricular nucleus (PVN; corresponding to Plates 26–28 in Paxinos and Watson [42]) and ventromedial nucleus of the hypothalamus (VMH; corresponding to Plates 30–32 in Paxinos and Watson [42]). These areas were chosen because they could be reliably defined in both adult and infant brains [15]. Although BDNF mRNA labeling was also found in the thalamus in both species, difficulty in determining the boundaries of specific thalamic regions in the infant brain, especially at 1 day or 1 week of age, prohibited us from reliably measuring BDNF mRNA labeling in those areas. The density of BDNF mRNA was quantified bilaterally from three to four sections for each brain area per animal, and means were used for data analysis. Developmental changes of BDNF mRNA labeling for each species were analyzed using a two-way analysis of variance (ANOVA), with age and sex as between subject variables. Significant age effects were further evaluated using a Student–Newman–Keul's (SNK) post-hoc test.

Alternations in the number of mRNA-labeled cells and/or grain density per labeled cell may each or both contribute to the variations in the density of mRNA labeling [9]. To test this, we further counted the number of BDNF-mRNA labeled cells and the grain density per labeled cell in the PVN and basolateral nucleus of the amygdala (BLA) in prairie voles from emulsion-coated slides. The number of BDNF-mRNA labeled cells for each subject was counted bilaterally in three sections per area (the images of those sections on the autoradiograms were quantified for the BDNF mRNA expression). In addition, 10 cells were chosen stereologically on the same spot from each measured area on each side of the section, and the grain density per labeled cell was counted on the aforementioned three sections using the imaging program (NIH IMAGE 1.60). The means for the number of cells and the grain density per cell were analyzed by a two-way ANOVA followed by a SNK test. We also tried to perform the same analysis on montane voles. However, the emulsion-coded slides were over-exposed, and overlapped grains prevented us from reliably measuring the grain density per labeled cell.

3. Results

The in situ hybridization revealed a specific distribution pattern of BDNF mRNA labeling in the prairie and montane voles. BDNF mRNA labeling showed a neonatal appearance and a transient expression during development in both voles in a regionally specific manner. Several patterns were observed. In the hippocampal formation, especially in the dentate gyrus (DG; $F=18.98$, $P<0.001$ for prairie voles and $F=9.05$, $P<0.001$ for montane voles) and CA3 region ($F=32.15$, $P<0.001$ for prairie voles and $F=16.10$, $P<0.001$ for montane voles), the level of BDNF mRNA increased gradually during early development and reached a peak at weaning (3 weeks of age), followed by a subsequent decline to the adult level (3 months of age) (Figs. 1 and 2). This developmental pattern was similar in both species. In the medial nucleus (MeA; $F=7.16$, $P<0.001$ for prairie voles and $F=8.25$, $P<0.001$ for montane voles) or basomedial nucleus (BMA; $F=5.66$, $P<0.01$ for prairie voles and $F=4.60$, $P<0.01$ for montane voles) of the amygdala, the level of BDNF mRNA expression showed a slight increase with some fluctuations in early development, followed by a significant decline to the adult levels (Fig. 3A,B). This decline took place in montane voles when they were 2 weeks of age but did not occur in prairie voles until they reached 3 weeks of age. In the cingulate cortex (Cg), BDNF mRNA labeling showed a transient increase in the second and third postnatal weeks during development then decreased to the adult level in prairie voles ($F=14.08$, $P<0.001$), but persisted throughout the course of development in montane voles (Fig. 3D). In the PVN, the level of BDNF mRNA labeling persisted until weaning, followed by a significant increase in both species ($F=16.67$, $P<0.001$ for prairie voles and $F=10.61$, $P<0.001$ for montane voles; Fig. 3E). Finally, in the VMH, the level of BDNF mRNA labeling showed a postnatal increase followed by a significant decline (Fig. 3F). In prairie voles, the decline represented a two-step process: first at 2 weeks of age and then again after weaning ($F=27.11$, $P<0.001$). In montane voles, however, such a decline occurred at weaning and was sustained thereafter ($F=7.09$, $P<0.001$). In general, no sex differences were detected in the BDNF mRNA labeling in either species. Due to the fact that the BDNF in situ hybridization was processed separately for each species, a direct comparison of the same age groups between the two species was not performed.

We also counted the number of BDNF-mRNA labeled cells and the grain density per labeled cell in the basolater-

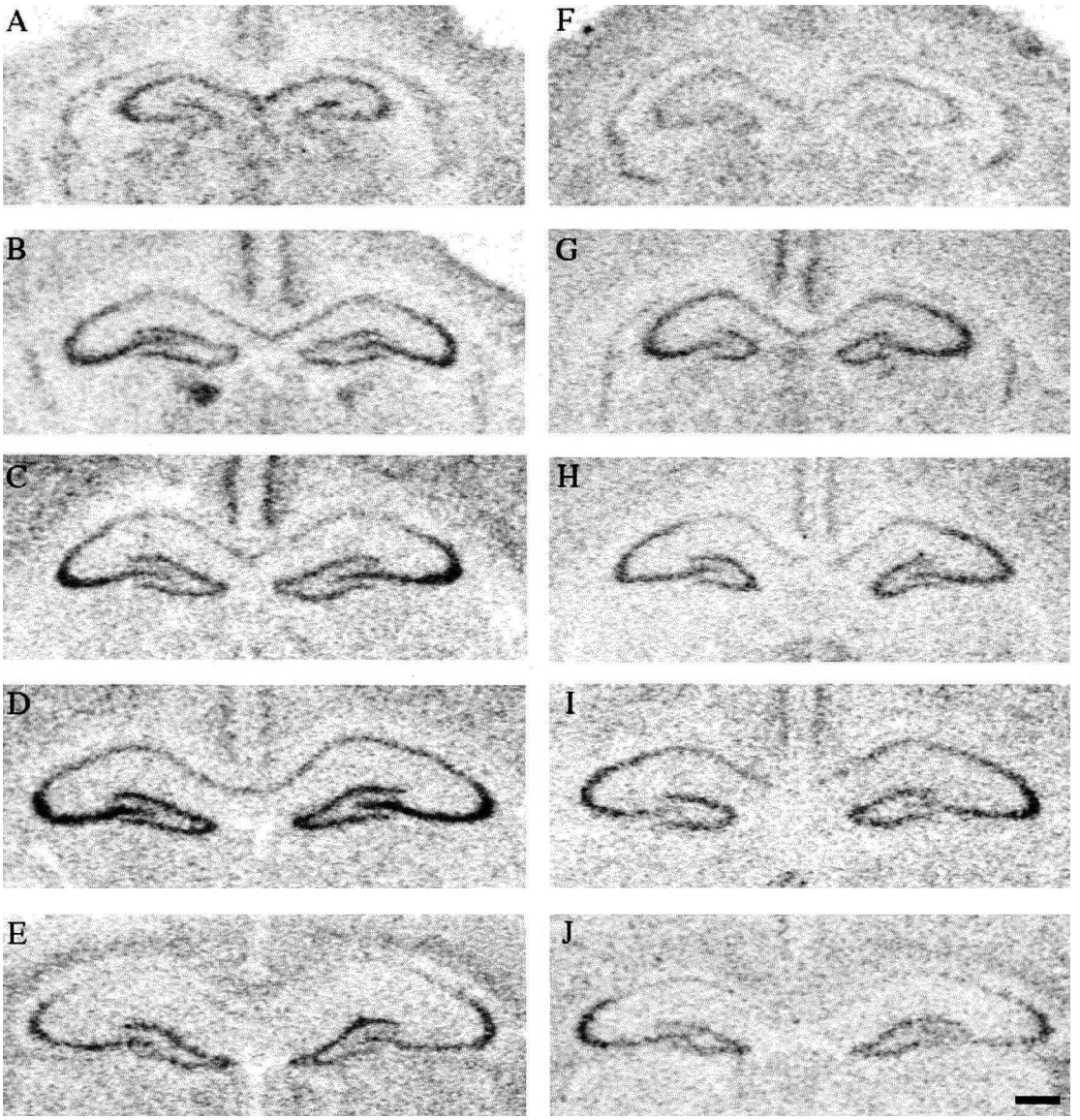

Fig. 1. Photomicrographs from the autoradiograms displaying BDNF mRNA labeling in the hippocampal formation of prairie voles (left) and montane voles (right) on the day of litter birth (A,F) or at 1 week (B,G), 2 weeks (C,H), 3 weeks (D,I), or 3 months of age (E,J). Scale bar=1 mm.

al nucleus of the amygdala (BLA) and PVN in prairie voles from emulsion-coated slides. Because of the overlapped and diffused grains, counting the cells and the grain density per cell could not be performed accurately for the 1-day-old subjects. Therefore, the analysis was only performed on the other developmental groups. In the BLA, the number of BDNF mRNA-labeled cells showed a gradual increase in early development until weaning ($F=46.61$, $P<0.001$). Prairie voles at 2 weeks of age had more BDNF mRNA-labeled cells than 1-week old voles but fewer than 3-week-old ones (Figs. 4 and 5A). At 3 months of age, the cell number was reduced significantly. The grain density per labeled cell in BLA also varied during development. The 1-week-old voles had a lower grain density per labeled cell than adults, which, in turn, had a lower grain density than the voles at 2 or 3 weeks of age ($F=27.72$, $P<0.001$; Fig. 5B). In the PVN, voles at different age groups did not differ significantly in the number of BDNF mRNA-labeled cells (Fig. 6C). However, voles at 3 weeks of age had grain density per labeled cell higher than younger voles but lower than the voles at 3 months of age ($F=47.20$, $P<0.001$; Figs. 5D and 6).

4. Discussion

In the present study, using in situ hybridization, we examined the ontogenetic pattern of BDNF mRNA expression in selected areas in the forebrain of prairie and montane voles. Our data reveal a neonatal appearance and a transient expression of BDNF mRNA labeling in the developing vole brains. In addition, our data indicate some

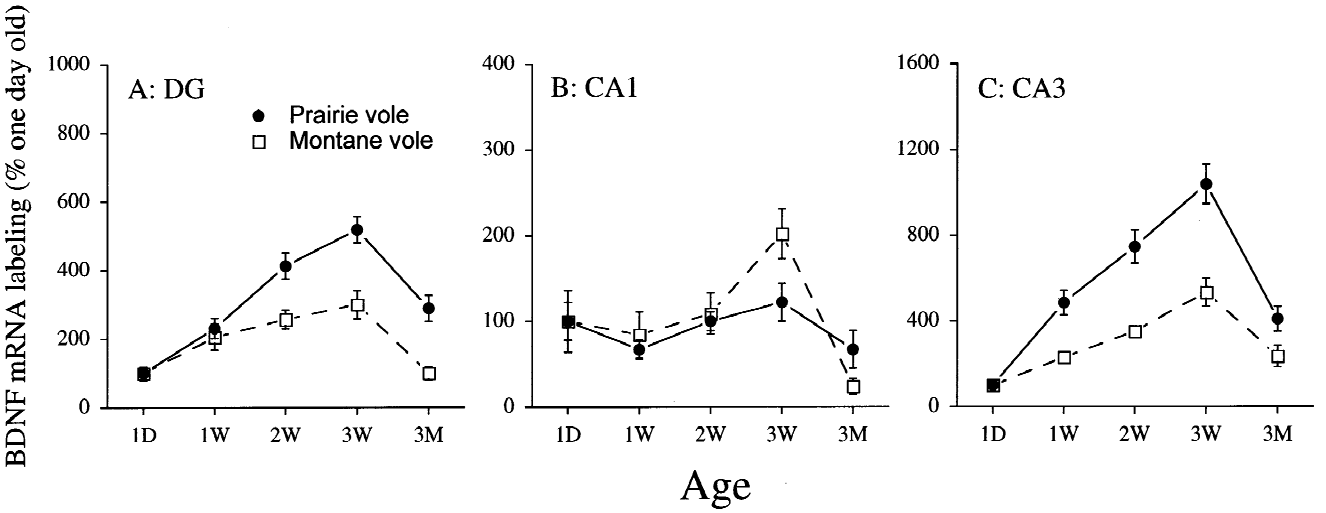

Fig. 2. The ontogenetic pattern of BDNF mRNA labeling in the dentate gyrus (DG; A), CA1 (B) and CA3 (C) regions of the hippocampal formation of prairie voles (solid line) and montane voles (dotted line). Data are presented as the percentage of changes relative to subjects at 1 day of age for each area per species. No sex differences were detected. Age: 1D, 1 day; 1W, 1 week; 2W, 2 weeks; 3W, 3 weeks; and 3M, 3 months.

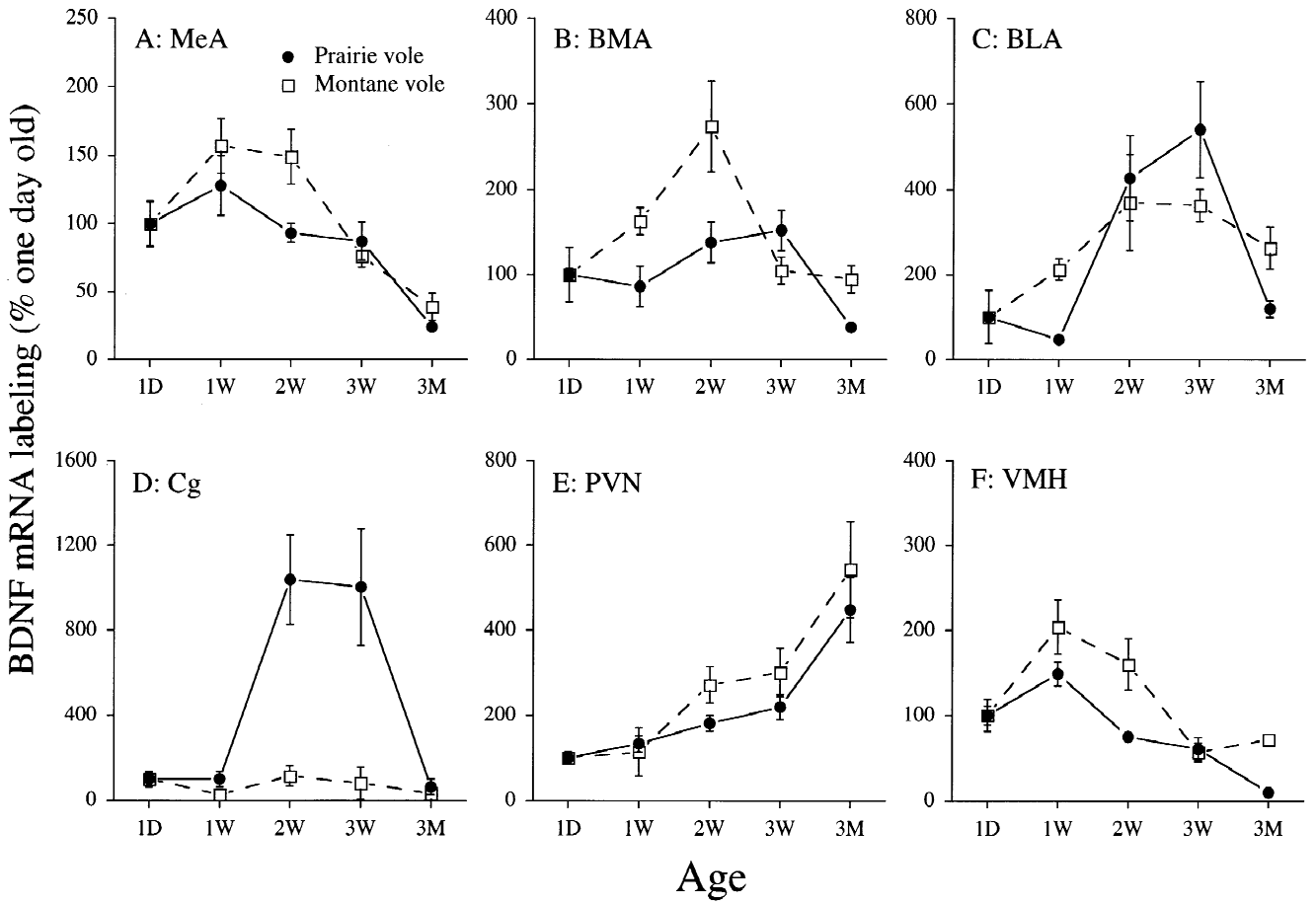

Fig. 3. The ontogenetic pattern of BDNF mRNA labeling in the medial nucleus (MeA; A), basomedial nucleus (BMA; B) and basolateral nucleus (BLA; C) of the amygdala, as well as in the cingulate cortex (Cg; D), paraventricular nucleus (PVN; E) and ventromedial nucleus (VMH; F) of the hypothalamus of prairie voles (solid line) and montane voles (dotted line). Data are presented as the percentage of changes relative to subjects at 1 day of age for each area per species. No sex differences were detected. Age: 1D, 1 day; 1W, 1 week; 2W, 2 weeks; 3W, 3 weeks; and 3M, 3 months.

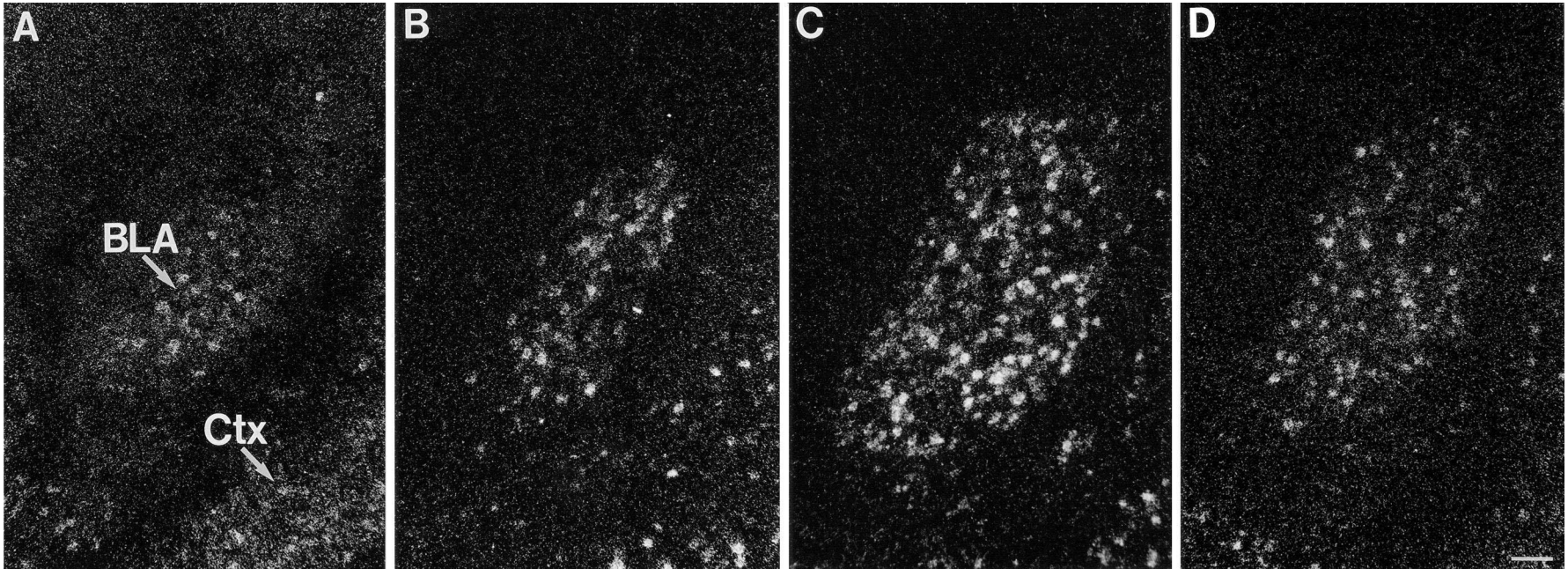

Fig. 4. Photomicrographs displaying BDNF mRNA-labeled cells in the basolateral nucleus of the amygdala (BLA) of prairie voles at 1 week (A), 2 weeks (B), 3 weeks (C), or 3 months of age (D). Ctx, cortex, Scale bar=10 μm.

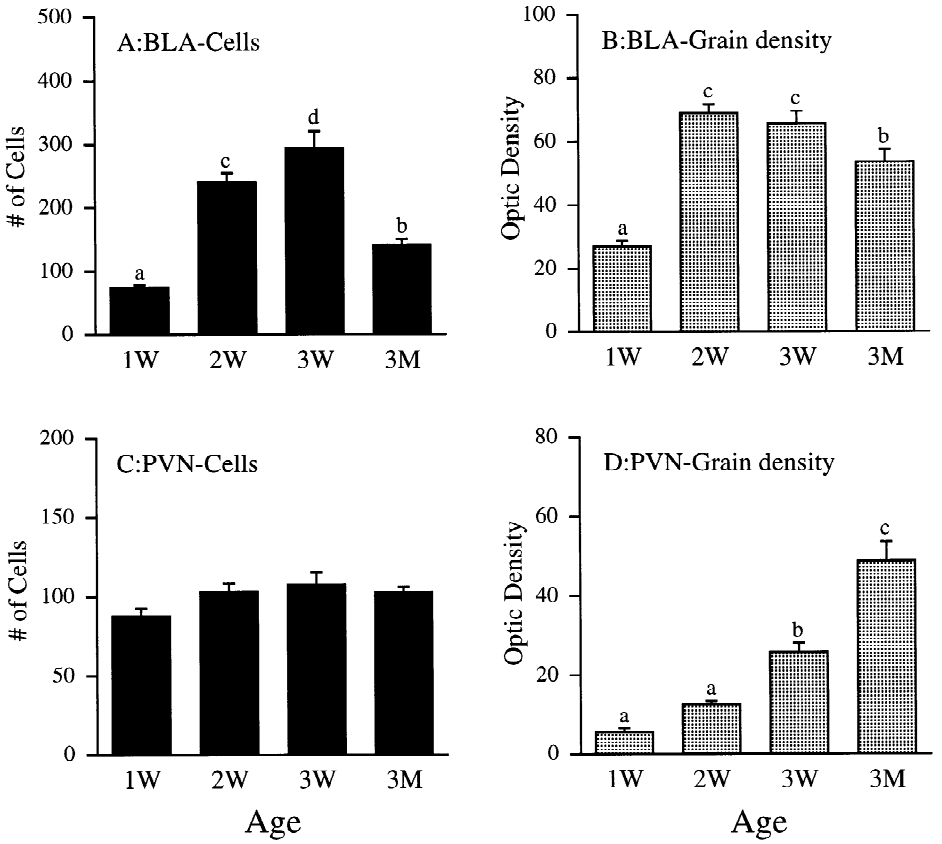

Fig. 5. Differences in the number of BDNF mRNA-labeled cells and grain density per labeled cell in the basolateral nucleus of the amygdala (BLA; A,B) and paraventricular nucleus of the hypothalamus (PVN; C and D) of prairie voles at 1 week (1W), 2 weeks (2W), 3 weeks (3W), or 3 months (3 M) of age. Data were analyzed by a two-way ANOVA followed by a Student–Newman–Keul's post hoc test. The alphabetic letters represent significant age differences. Bars=means±S.E.M.

differences in the developmental pattern of BDNF mRNA expression in some brain regions between two species that display different life strategies and social behaviors.

4.1. Developmental changes in BDNF mRNA expression

BDNF mRNA expression showed a neonatal appearance and varying expression over the course of postnatal development in both prairie and montane voles. Several general patterns were observed. Differences in BDNF mRNA expression in the brain between infants and adults indicate differences in the level of BDNF synthesis and availability, suggesting that the function of BDNF in developing voles may be quite different from that of adult animals.

A neonatal appearance and varying expression of BDNF mRNA labeling during development has also been reported in rats [12,15,45]. High levels of BDNF mRNA labeling during development may indicate the importance of BDNF in regulating neuronal growth, differentiation and functional organization at critical stages during development. On the other hand, a marked decrease in BDNF mRNA expression in adulthood may indicate a decrease of neuronal dependency on BDNF after maturation [36], even though BDNF may still have maintenance and survival functions on the neuronal populations [34]. Finally, regional differences in BDNF mRNA ontogeny may reflect different requirements of distinct neuronal groups for BDNF during development.

What is the mechanism underlying the ontogenetic changes of BDNF mRNA expression? At least two mechanisms could be proposed: (1) genesis or death of neuronal cells that synthesize BDNF and (2) changes in the rate of BDNF expression of individual neurons. Although the peak of changes in the second or third postnatal week during development seems too late to be explained by the major wave of neurogenesis (which occurs prenatally), our data indicate that changes in the number of cells that synthesize BDNF, at least in the BLA of the prairie voles, contributed to the developmental changes in BDNF mRNA expression. Our data in both the PVN and BLA in prairie voles also indicate that the grain density per labeled cell varied during development, reflecting changes in the ability of individual cells to synthesize BDNF. Certainly, this could also account for the increase or decrease in the number of cells expressing BDNF, as more cells would rise above or drop below a detection threshold as the level of BDNF mRNA changed.

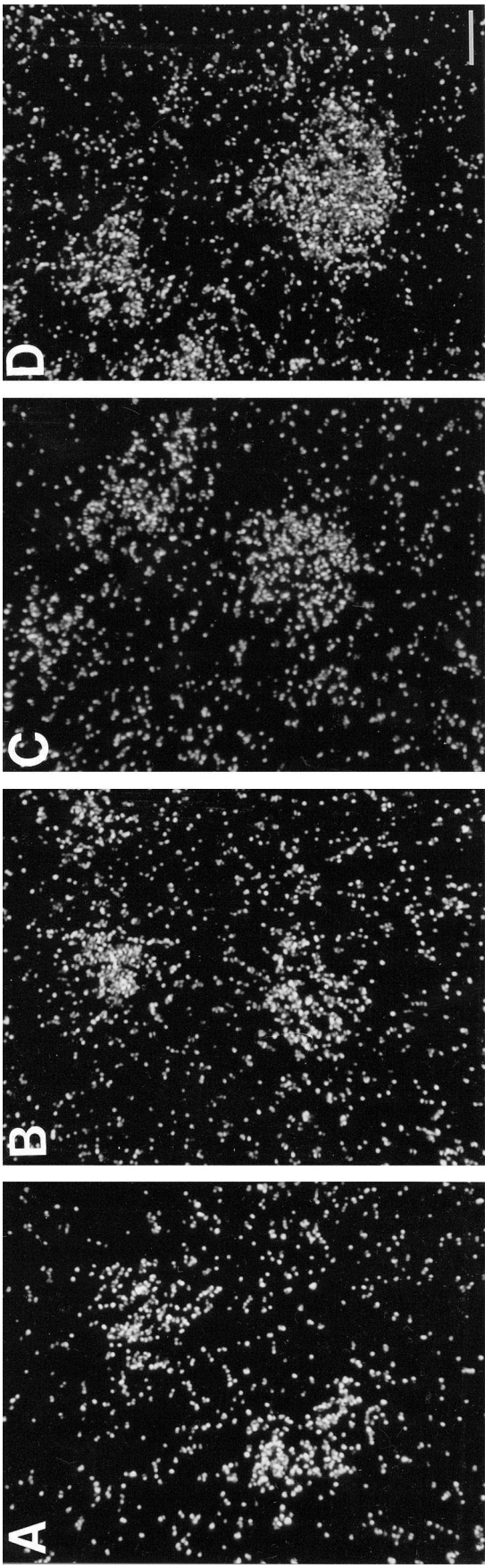

Fig. 6. Photomicrographs displaying grain densities per BDNF mRNA-labeled cell in the paraventricular nucleus of the hypothalamus of prairie voles at 1 week (A), 2 weeks (B), 3 weeks (C), or 3 months of age (D). Scale bar=1 μm.

In the present study, male and female voles showed a similar pattern of BDNF mRNA expression during the postnatal development in selected brain regions of both species. However, in a recent study using ribonuclease protection assays, the developmental profile of BDNF mRNA in the hypothalamus and the cerebral cortex was found to be sex- and region-specific in rats [28]. It is not yet clear how to interpret this discrepancy. Differences in the ontogenetic pattern of BDNF expression between voles and rats may represent species-specific mechanisms through which specific neural and behavioral development could be influenced. Alternatively, it may be due to differences in the sensitivity of the techniques employed for each study.

4.2. Species-specific pattern of BDNF mRNA expression

Although both species displayed varying expressions of BDNF mRNA labeling in the brain, prairie and montane voles showed some subtle differences in the developmental pattern of regional BDNF mRNA labeling. A species difference was found for the time that BDNF mRNA labeling reached adult levels. In several brain regions, such as the VMH and BMA, BDNF mRNA labeling in prairie voles exhibited a rapid decline after the third postnatal week (weaning) and approached the adult levels at 3 months of age. In montane voles, however, such a decline occurred after the second postnatal week, and thereafter, the level of BDNF mRNA was sustained into adulthood. These data indicate that montane voles reached to the adult pattern of BDNF mRNA expression earlier than did prairie voles, suggesting that the two species might be at different functional stages while at the same age during development.

The hypothesis that the promiscuous voles develop more rapidly than monogamous voles [19] is supported by some data from previous studies. Promiscuous litters eat solid food, terminate nipple attachment, and wean from the natal nest earlier relative to monogamous litters [37,38]. In addition, promiscuous pups show a faster rate of overall brain and cerebral growth and a slightly earlier peak of thymidine kinase activity, suggesting an accelerated rate of brain development, compared to monogamous pups [19]. Our data indicate an earlier appearance of the adult pattern of BDNF mRNA expression in montane than in prairie voles, providing further evidence to support the notion that the promiscuous voles develop faster than monogamous voles. As BDNF has broad actions on development and plasticity for a variety of neuronal populations [11,13,26] and BDNF expression has been associated with brain maturation [15,34], it is possible that BDNF may play a role in determining the rate of neural and behavioral development. Conversely, a rapid rate of brain development may lead to an early rise in BDNF expression. The causal relationship between BDNF and brain development needs to be further studied.

Another species difference in the developmental pattern of BDNF mRNA expression was evident in the cingulate cortex. In montane voles the level of BDNF mRNA labeling persisted throughout the course of development, whereas in prairie voles the level of BDNF mRNA labeling displayed a transient increase followed by a significant decrease. It is interesting to note that a similar transient increase in BDNF mRNA was also found in the BLA of the prairie voles. The neural pathways in the vole brain are still unknown. In rats, the cingulate cortex is considered to be a part of the limbic system and has a complex array of reciprocal fiber connections with the BLA [47,52]. The cingulate cortex has been implicated in agonistic encounters in hamsters [33] while the amygdaloid complex plays an important role in modulating pair bond formation and male parental behavior associated with the monogamous life strategy in prairie voles [29,30,54]. It is possible that this species difference in regional BDNF synthesis may contribute to differential neuronal development, which, in turn, may lead to different functional significance between monogamous and promiscuous voles. Nevertheless, this speculation needs to be examined in further studies.

5. Conclusions

In the present study, we found that BDNF mRNA expression showed an early appearance and a transient expression in the developing vole brain, which is consistent with earlier reports in rats [12,15,45]. In addition, promiscuous montane voles and monogamous prairie voles differed in the ontogenetic pattern of BDNF mRNA expression in some brain regions, with the former reaching to the adult pattern of BDNF expression sooner than the latter. Although these data provide evidence to support the hypothesis that promiscuous voles develop more rapidly than monogamous voles [19], the genetic factors and/or environmental pressures that brought about this developmental difference between the two species of voles are still unknown. In addition, the functional significance of BDNF on brain and behavioral development needs to be further studied.

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