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Localization of Brain-Derived Neurotrophic Factor and Neurotrophin-3 RNA Transcripts in the Brain of Reeler, Weaver and *pcd* Mutant Mice

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Summary. **The pattern of BDNF and neurotrophin-3 (NT-3) gene expression was analyzed in the cerebellum of wild-type (+/+), reeler (*rl/rl*), weaver (*wv/wv*) and 'Purkinje cell degeneration' (*pcd/pcd*) mice by *in situ* hybridization histochemistry. [^{32}P] Labeled synthetic oligonucleotide DNA probes were used, complementary to bases 219-266 and 777-824 of the published mouse BDNF cDNA sequence and to bases 91-138 and 235-288 of the rat NT-3 genomic sequence. Previous developmental studies in rat cerebellum point to a possible involvement of NT-3 in early migratory and synaptogenetic events and of BDNF as a maintenance factor at later stages. In the wild-type mice, strong mRNA hybridization signals were obtained for both BDNF and NT-3 in the cerebellar granule cell layer. Reeler and weaver mice showed remarkable reductions for both BDNF and NT-3 mRNA hybridization signals in the cerebellum, which is readily explainable by the genetically-determined granule cell loss. The cerebellum of *pcd* mice, which primarily lose Purkinje cells, showed a slight reduction in BDNF and NT-3 mRNAs. Reeler homozygotes also showed a substantial decrease of the BDNF RNA message in the pyramidal cell layer of hippocampal CA1 field; in the cerebral cortex, the lamination was not as evident as that in normal mice. The changes recorded in BDNF and NT-3 RNA messages in these mutants are consistent with the localization of nerve cell losses. Nonetheless, their roles and possible involvement in early developmental or degenerative events remain open. These results could also suggest a coordinated down-regulation of these genes subsequent to the degeneration of Purkinje cells.**

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INTRODUCTION

Several trophic factor systems are known to be involved in various stages of cerebellar ontogeny and maintenance. These include two members of the neurotrophin family: brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3).

BDNF is a dimeric protein of 13.5-kDa subunits that is produced mainly in the CNS.[1] It has been shown to act through the *trk*B tyrosine kinase receptors: the *trk* proto-oncogene is transcribed only in neural crest-derived components of the nervous system;[2] *trk*B, a gene structurally related to *trk*, is expressed in embryonic and adult nervous systems and has been identified as the receptor for BDNF.[3] BDNF treatment of primary cerebellar cultures induces RNA production of *Pax*-2, *Pax*-3 and *Pax*-6 genes that encode sequence-specific DNA binding transcripiton factors expressed in embryonic development of the nervous system.[4] Gene expression of BDNF has been detected by *in situ* hybridization histochemistry in areas where donor fetal Purkinje and granule cells had been earlier transplanted in the cerebellum of adult rats.[5]

Previous studies in rats[6] suggest a local delivery and a role for NT-3 in early migratory events during postnatal cerebellar ontogeny as well as a possible involvement in the establishment of early synaptic contacts. BDNF, on the other hand, might play a role at a later stage, perhaps as a trophic factor essential

for sustained survival. The aim of the present study was to analyze by *in situ* hybridization histochemistry the anatomical pattern of BDNF and NT-3 gene expression in the brains of mice with mutations affecting the survival of specific cerebellar neuron types.

In particular, reeler mutant mice (*rl/rl*) have a systematic malposition of neuron classes in the cerebellum.[7,8,9] The typical organization and lamination of the cerebellar cortex is altered.[7,8,10] The reeler cerebellum is small in size and contains a reduced complement of granule cells.[7,8] The salient architectonic perturbation consists of a malpositioning of Purkinje cells, most of which lie heterotopically in or below the granule cell layer.[11] In the cerebral cortex, the laminar segregation of postmigratory cells proceeds abnormally during development, and in the adult the polymorphous cell population is superficial rather than deep in relation to the pyramidal cell classes.[9] The plexiform layer is absent, and large pyramidal neurons are located superficially, whereas medium and small-sized pyramidal cells are concentrated in the depth of the cortex, and small stellate cells are intercalated between the large and medium-sized pyramidal cells.[9] In Ammon's horn, pyramidal cells are radially dispersed rather than forming a compact layer, and in the fascia dentata, granule cells are mixed with neurons of the hilus.[12]

The cerebellum of weaver mutant mice (*wv/wv*) is characterized by the failure of the majority of postmitotic granule cell precursors in the external germinal layer to emit axons and migrate to the internal granular layer, and by massive cellular death at the interface of the external germinal and the molecular layers during the first two weeks of postnatal life.[13,14] The cerebellum of adult weaver mutants is granuloprival.[15]

In 'Purkinje cell degeneration' mutant mice (*pcd/pcd*), there is a rapid degeneration of nearly all Purkinje cells beginning at 17 days of age.[16] Purkinje cell death occurs between the third and sixth weeks of life and is preceded by an abnormal retention of perikaryonal polysomes.[17]

MATERIALS AND METHODS

Animals

Reeler and *pcd* mutant mice of a B6C3Fe hybrid stock and weaver mutant mice of a B6CBA-A^{w-J}/A hybrid stock, along with corresponding wild-type control mice, were purchased from Jackson Laboratory (Bar Harbor, Maine). All animals were kept on a 12 hr dark-12 hr light cycle and provided with food and water *ad libitum*. For the present study, three male mice of each genotype were used at an age of 2 ~3 months. All experiments reported in this article were conducted in compliance with the guidelines of the National Institutes of Health for the care and use of laboratory animals for experimental procedures.

Tissue processing

Mice were decapitated and brains were rapidly removed from the cranium and frozen on dry ice. Coronal sections of the cerebrum and cerebellum (20 μm thick) were obtained in a cryostat (Leitz model 1720), thaw-mounted on acid-cleaned, gelatin-coated slides, and kept at −20℃ until use. Two consecutive sections were mounted on each slide.

In situ hybridization histochemistry

In situ hybridization histochemistry was essentially performed as previously described.[6] Briefly, tissue sections were air-dried, fixed by immersion in 4% formaldehyde, 1×PBS (containing 2.6 mM KCl, 1.4 mM KH_2PO_4, 136 mM NaCl, 8 mM Na_2HPO_4) and incubated with predigested pronase (24 U/ml in 50 mM Tris-HCl pH 7.5, 5 mM EDTA). Finally, sections were dehydrated in graded aethanols (60, 80, 95 and 100%, 2 min each) and air-dried.

Two oligonucleotide probes were used for each neurotrophin, complementary to bases 219-266 and 777-824 of the mouse BDNF mRNA sequence[18], and to bases 91-138 and 235-288 of the rat NT-3 genomic sequence.[19] Oligonucleotides were 3' end-labeled using [α-^{32}P] dATP (3000 Ci/mmol, New England Nuclear) and terminal deoxynucleotidyltransferase (TdT, Boehringer Mannheim) to specific activities 0.9-2.0×10^4 Ci/mmol.

Labeled probes were diluted to a final concentration of 2-3×10^7cpm/ml in 0.6 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1×Denhardt's solution (0.02% ficoll, 0.02% polyvinylpyrolidone, 0.02% bovine serum albumin), 500 μg/ml yeast tRNA, 50% formamide, 10% dextran sulfate. Tissue was covered with 65 μl of hybridization solution, overlaid with Nescofilm coverslips, and incubated overnight in humid chambers at 42℃. Sections were then washed at 60℃ in 0.6 M NaCl, 20 mM Tris-HCl pH 7.5, 1 mM EDTA, for 3 hr with four buffer changes. Tissue was dehydrated in 70% and 95% Aethanols containing 0.3 M ammonium acetate pH 7.0. Hybridized tissue sections were finally exposed to β-max autoradiographic films (Amersham, U. K.) at −70℃ with intensifying screens.

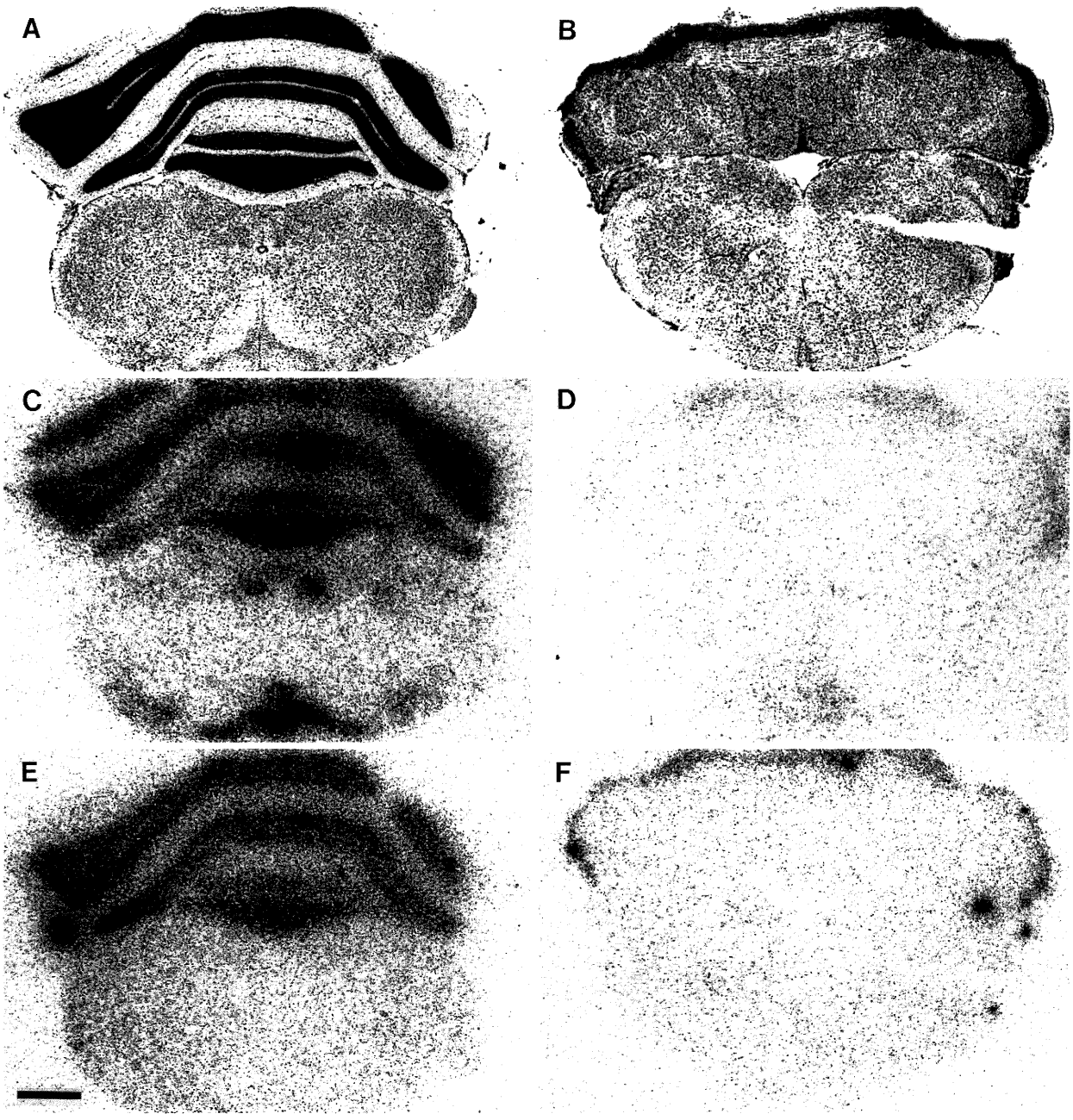

Fig. 1. *In situ* hybridization histochemistry with [^{32}P] labeled oligonucleotide probes to detect mRNAs for BDNF (**C, D**) and NT-3 (**E, F**) in coronal sections of the cerebellum from wild-type (**A, C** and **E**) and homozygous reeler mutant mice (**B, D** and **F**). Fields (**A**) and (**B**) show conventional histological sections stained with cresyl violet. In normal mice, both BDNF and NT-3 hybridization signals are largely confined to the granule cell layer. In the reeler cerebellum, there is only a small amount of signal in the outermost part of the cerebellum, corresponding to displaced cells. Scale bar = 1 mm

RESULTS

In the cerebellum of wild-type mice, the strongest hybridization signal for BDNF and NT-3 mRNA was seen in the granule cell layer (Fig. 1A, C and E). Since most of the volume of granule cell somata, the diameter of which is about 5 μm, is occupied by the

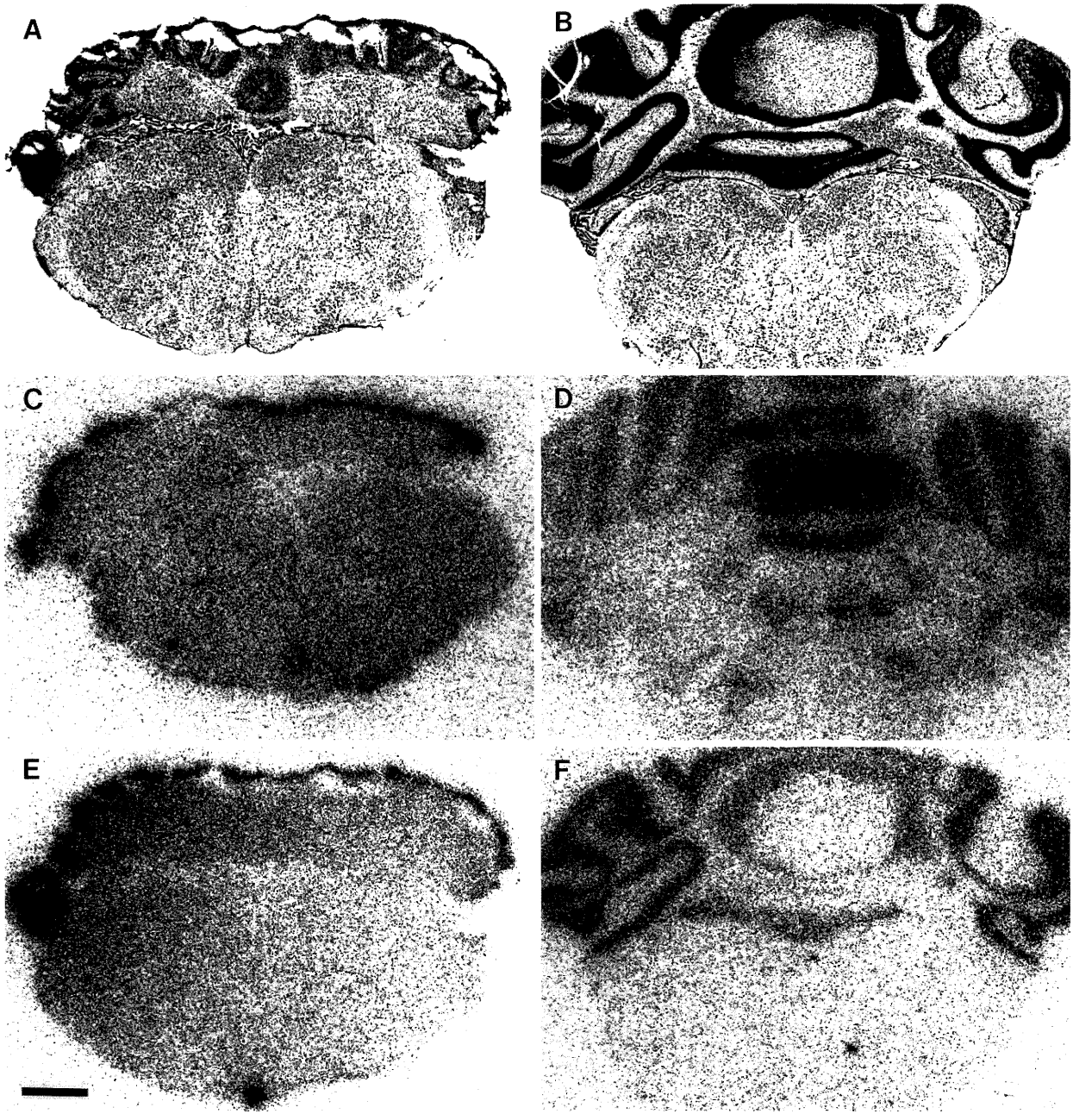

Fig. 2. *In situ* hybridization histochemistry with [^{32}P] labeled oligonucleotide probes to detect mRNAs for BDNF (**C, D**) and NT-3 (**E, F**) in coronal sections of the cerebellum from weaver (**A, C** and **E**) and *pcd* mutant mice (**B, D** and **F**). Fields (**A**) and (**B**) show conventional histological sections stained with cresyl violet. In the weaver cerebellum, the hybridization signal for both BDNF and NT-3 is substantially decreased inside the cerebellar parenchyma. In the cerebellum of the *pcd* mutant, which loses Purkinje cells, there is a certain reduction in signal intensity for both neurotrophins in the granule cell layer. Scale bar=1 mm

cell nucleus, the thickness of the perikaryon that would be expected to contain the RNA message and the thickness of the tissue sections (20 μm) would theoretically overlap in terms of silver grain distribution. Both of the oligonucleotide probes that were used for each of the neurotrophins studied gave

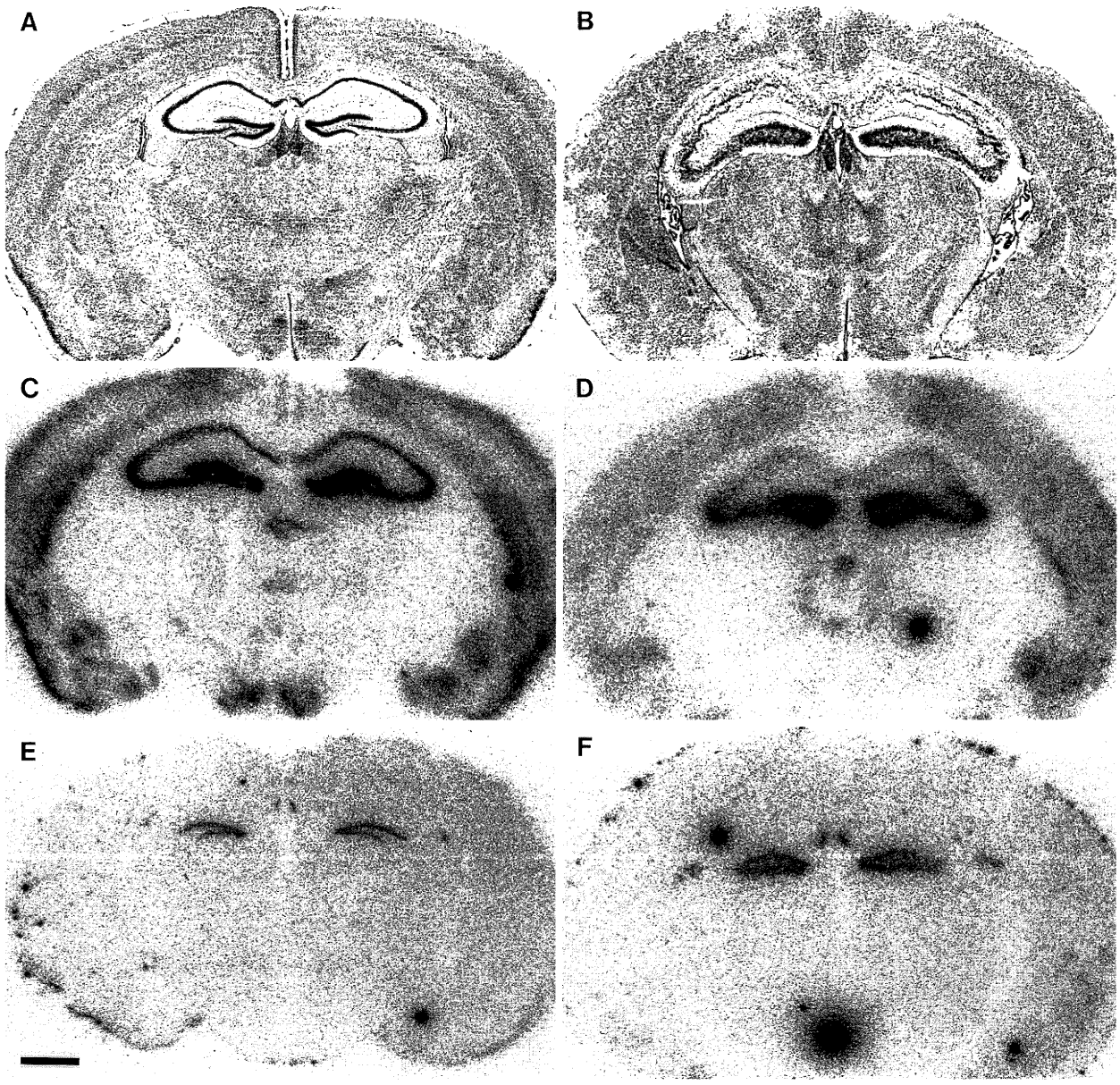

Fig. 3. *In situ* hybridization histochemistry with [^{32}P] labeled oligonucleotide probes to detect mRNAs for BDNF (**C, D**) and NT-3 (**E, F**) in coronal sections of wild-type (**A, C** and **E**) and reeler (**B, D** and **F**) forebrains. Fields (**A**) and (**B**) show conventional histological sections stained with cresyl violet. In normal mice, a strong BDNF mRNA hybridization signal is seen in pyramidal and granule neuron layers; the NT-3 signal is mostly confined to the dentate gyrus. In the reeler hippocampus and cortex, there is a mixing of layers with no clear-cut demarcation, and an overall reduction of BDNF mRNA in the field CA1. Scale bar = 1 mm

similar patterns of hybridization.

The reeler cerebellum was cytoarchitectonically disorganized and granule cells were arranged in one thin layer without the typical foliation pattern. (Fig. 1B). Purkinje cells were dispersed to various sites throughout the cerebellum, including the subcortical white matter, and interspersed within the area of the deep cerebellar nuclei. A diffuse, background-level hybridization signal for BDNF and NT-3 mRNA was seen in the cerebellum (Fig. 1D and F).

In the weaver cerebellum, the majority of granule cells were absent (Fig. 2A). The overall hybridiza-

tion signal of mRNAs for both neurotrophins in the autoradiographic films was markedly reduced in the cerebellar cortex (Fig.2C and E). Purkinje cells were virtually absent from the cerebellum of *pcd* mutants (Fig. 2B). Hybridization for both BDNF mRNA (Fig. 2D) and NT-3 mRNA (Fig.2F) was seen in the granule cell layer of weaver mutants, but the hybridization signal appeared less intense compared with wild-type mice (which are shown in Fig. 1C and 1E).

A strong hybridization signal for BDNF mRNA was seen in hippocampal fields CA 1-4 and the fascia dentata of normal mice (Fig.3C). The distribution of NT-3 mRNA was much more restricted and comprised an area corresponding to the granule cells of the dentate gyrus and a small group of pyramidal cells in field CA2 (Fig.3E). In the reeler hippocampal formation, the hybridization signal for both BDNF and NT-3 mRNA was present (Fig.3D and F). However, in the coronal plane there was a reduction of BDNF mRNA in the field CA1 and a dispersion of the autoradiographic labeling signal in the remaining fields of Ammon's horn as opposed to the compact cellular lamina seen in wild-type mice. Additionally, the fascia dentata, the granule cell layer and the hilar region appeared intermixed (Fig. 3B, D and F).

A hybridization signal for BDNF was seen in the cerebral cortical fields of wild-type mice, including the piriform cortex (Fig. 3C). In the reeler cortex, the overall BDNF mRNA signal was diffuse (Fig.3D), and no clear-cut separation of layers could be ascertained as in the normal situation (which is shown in Fig. 3C). Such a mixing of layers is known to be an aspect of the histological phenotype in the forebrain of the reeler mutant.

DISCUSSION

We studied the distribution of the mRNA transcripts of two neurotrophins, BDNF and NT-3, in normal and neurologically mutant mice. We found that, in normal mice, these genes are expressed in the cerebellar granule cell layer, hippocampal formation, and cerebral cortex. Our results on the normal anatomical localization of BDNF and NT-3 mRNAs in the cerebellum and forebrain of wild-type are in agreement with previous studies in rats.[6,20]

The signal is decreased when the same cells degenerate in mutants. The cellular localization in mutants correlates with their known anatomical deficits. The genes are expressed by surviving subsets of granule cells when Purkinje cells are the primary target of a mutation, as in the case of the *pcd* mutation. In other words, the genes are expressed by neurons that are connected presynaptically to the cells that degenerate.

Mutant mice with selective loss of specific neuronal populations are particularly useful in mapping the anatomical expression of genes in the brain. The expression can be dissected by subtraction of the cells that degenerate in each mutant. In normal rats, NT-3 expression peaks early during the two postnatal weeks, whereas BDNF mRNA peaks later around postnatal day twenty.[6]

In a different study,[21] a highly sensitive immunoassay was used to measure the levels of the protein product of another neurotrophin, nerve growth factor (NGF), in the brains of weaver, reeler and *pcd* mutant mice. A significant reduction was observed in the reeler and weaver cerebellum, but not in the *pcd*, leading those authors to postulate that granule cells may be involved in regulating cerebellar NGF levels.

The expression of these neurotrophins could theoretically depend on transacting elements produced by other neuronal cell types. For example, reduced expression by granule cells in the *pcd* mutant suggests that elimination of their postsynaptic Purkinje cells might retrogradely influence gene expression by granule cells, at least on qualitative assessment. These findings are important because they provide evidence that in the mutants studied, primary or secondary effects of degeneration in the cerebrum and cerebellum may alter expression of BDNF and NT-3 by various neuron populations.

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