Characterization of the Peripheral Neuropathy in Neonatal and Adult Mice That Are Homozygous for the Fatty Liver Dystrophy (fld) Mutation*

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From the Departments of †Biochemistry and Molecular Biophysics, ‡Pathology, and $$Medicine, Washington University School of Medicine, St. Louis, Missouri 63110, ¶The Jackson Laboratory, Bar Harbor, Maine 04609, and the **Department of Pathology, Tufts University Schools of Medicine and Veterinary Medicine, Boston, Massachusetts 02111

In a recent report (Langner, C. A., Birkenmeier, E. H., Ben-Zeev, O., Schotz, M. C., Sweet, H. O., Davidson, M. T., and Gordon, J. I. (1989) J. Biol. Chem. 264, 7994–8003), we characterized the early developmental phenotype of mice that were homozygous for the autosomal recessive fatty liver dystrophy (fld) mutation. Shortly after birth, these mice can be distinguished from their +/? littermates by large pale livers, hypertriglyceridemia, elevations in hepatic apolipoprotein A-IV and apoC-II mRNA levels, and tissue-specific decreases in lipoprotein lipase and hepatic lipase activities. These traits resolve by the early weaning period. We have now characterized a second feature of this mutation: a peripheral neuropathy that becomes manifest by an abnormal gait at the end of the second postnatal week and persists through adulthood. Electron microscopic studies of sciatic nerves from 4-day-1-year-old fld/fld mice demonstrated a variety of abnormalities including thin, poorly compacted myelin sheaths, active myelin breakdown, and enlarged Schwann cell mitochondria and nuclei. Western blot analysis of sciatic nerve homogenates prepared from 1 to 3-month-old fld/fld mice and their +/? littermates indicated that homozygous animals have striking reductions in two peripheral nerve myelin-associated proteins, P1 and P2. The steady-state level of apoE, a protein induced during nerve regeneration, is markedly elevated. Furthermore, two axon-specific proteins, neurofilament 200K and growth-associated 43 protein, display altered expression in adult fld/fld sciatic nerves. High performance thin-layer chromatography revealed deficiencies in phospholipids, glycosphingolipids, and some neutral lipids in fld/fld sciatic nerves harvested during the first several months of life (compared to their +/? littermates). Cholesterol esters were elevated in homozygotes. By contrast, no differences in brain lipids were noted between fld/fld animals and their +/? littermates. These data suggest that the fld mutation is associated with an abnormality of myelin formation (demyelination) as well as demyelination and axonal degeneration that persists despite apparent resolution of the neonatal hypertriglyceridemia and associated lipase abnormalities. These findings establish the fld/fld mouse as an excellent model system for analyzing homeostatic mechanisms that modulate lipid metabolism in newborn mice and for examining the pathogenesis of peripheral neuropathies associated with dyslipidemias.

We have recently analyzed (Langner et al., 1989) some of the underlying biochemical abnormalities that occur as a result of a recently described autosomal recessive mutation affecting mice, known as fatty liver dystrophy (fld) (Sweet et al., 1988). Within the first few days of postnatal life, several-fold elevations occur in hepatic and serum triglyceride levels (compared to age- and gender-matched +/? littermates). These are accompanied by a 100-fold increase in hepatic apoA-IV mRNA levels and a 6-fold increase in the apoC-II mRNA concentration. During the suckling period, tissue-specific deficiencies in lipoprotein lipase occur: activity is reduced ~16-fold in white adipose tissue and <2-fold in heart, but is not altered in brown adipose tissue or liver. Hepatic lipase activity and mRNA levels are also reduced ~6-fold in serum and 2-fold in liver, respectively. These abnormalities resolve during the weaning period (postnatal days 14–28). This resolution is not delayed by prolongation of suckling, and the lipid abnormalities cannot be elicited in 4–6-week-old fld/fld mice by a high fat diet. The fld locus is distinct from loci encoding lipoprotein lipase, hepatic lipase, and apoA-IV and those responsible for the combined lipase deficiencies in cld/cld and W/Wc mice. Together, these findings suggested that the fld mutation was associated with developmentally programmed, tissue-specific defects in the neonatal expression of lipoprotein and hepatic lipase activities.

We also noted that fld/fld mice have an unsteady gait that appears in the suckling period and persists throughout life (Sweet et al., 1988; Langner et al., 1989). This raised the question of whether homozygotes have a peripheral neuropathy associated with myelin abnormality.

In the central and peripheral nervous systems (CNS1 and PNS, respectively) of mice and rats, myelination commences...
shortly after birth and involves three phases: (i) an initial slow increase in myelin during the first few postnatal days, (ii) a period of rapid myelination during the next 2–3 weeks, and (iii) a leveling off to steady-state levels as animals reach adulthood (Heape et al., 1986). Morphological studies of rat sciatic nerves indicated that the number of myelin spirals around axons increases from the first postnatal day (where one-half to one spiral turn is present around only the largest axons) to postnatal day 16, when mature compact myelin sheaths are encountered (Webster, 1971). Ontological changes in myelination are also accompanied by changes in the steady-state levels of myelin-specific mRNA, protein, and lipid species. Myelin protein markers include P0, P1, and P2 (Wiggins et al., 1975; Hahn et al., 1987). Galactosphingolipids (cerebrosides and sulfatides) are first detectable just prior to the onset of myelination and become more prevalent as peripheral nerves mature (Eccleston et al., 1987). Phosphatidylethanolamine, sphingomyelin, and cholesterol levels increase, whereas phosphatidylcholine and phosphatidylethanolamine concentrations decrease with increasing age in rat and mouse peripheral nerves and brains (Heape et al., 1986, Juguelin et al., 1986; Eng and Noble, 1968, Horrocks, 1968). Thus, mature myelin can be distinguished from immature myelin by its high content of cerebrosides, sulfatides, cholesterol, and phosphatidylethanolamine. The ratios of these lipid species to phosphatidylcholine serve as sensitive indicators of nerve development (Norton and Cammer, 1984).

Once mature myelin is formed, it is maintained and repaired as necessary. During nerve regeneration, the concentrations of cholesterol, cerebrosides, sulfatides, sphingomyelin, and phosphatidylethanolamine decrease, and myelin-specific proteins are degraded (Norton and Cammer, 1984). This is accompanied by an increase in the concentration of phosphatidylcholine, reflecting the relative decrease in myelin and subsequent enrichment of Schwann cells or macrophages (Hofteig et al., 1982). Under normal conditions, only myelin present in the PNS has the capacity to regenerate (Rambn y Ignatius, 1990). ApoE synthesis and export by nonmyelinating Schwann cells or macrophages is accompanied by an increase during myelin breakdown, regardless of the presence or absence of significant axonopathy (Boyles et al., 1986; Ignatius et al., 1986; Snipes et al., 1986; Gelman et al., 1987). This facilitates cholesterol transport to regenerating axonal growth cones and myelin sheaths, where it can be reused as the nerve is repaired and new myelin sheaths are formed (Boyles et al., 1989; Pitas et al., 1987; Ignatius et al., 1987).

Dysmyelination has been defined as a genetically determined disorder in myelogenesis leading to the production of an unstable form of myelin that is vulnerable to degeneration (Fosse, 1969). A number of mouse models have been described that have genetic abnormalities that produce dysmyelination. For example, myelin sheaths fail to mature in the PNS and CNS of quaking mice, resulting in a myelin phenotype normal +/? utters were anesthetized with Avertin (0.25 mg/g of body weight) (Jones and Krohn, 1960). Following transcardiac perfusion with 0.25 × and 0.5 × Karnovsky’s fixative (diluted with 0.1 M sodium cacodylate, pH 7.4), the brain and both sciatic nerves were dissected from each animal and stored in 0.5 × Karnovsky’s fixative for 24–48 h (Karnovsky, 1965). These tissues were then transferred to 0.1 M sodium cacodylate, pH 7.4, and subsequently embedded. Brain tissue was dehydrated through a series of graded alcohol solutions and xylene and embedded in paraffin. Eight-micrometer-thick sections of paraffin-embedded brain were stained using hematoxylin and eosin, Bielschowsky’s silver, and Luxol fast blue/periodic acid-Schiff stains. Sciatic nerves were postfixed in phosphate-buffered 2% osmium tetroxide containing 1.5% potassium ferricyanide, dehydrated in graded ethanol, and embedded in Spurr’s medium with propylene oxide as an intermediary solvent. One-micrometer-thick plastic sections were stained with toluidine blue and examined by light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips 200 electron microscope.

**Experimental Procedures**

**Animals**—All mice were produced and reared in the Mouse Mutant Resource colony at The Jackson Laboratory. Pregnant and lactating females as well as their weaned offspring were fed The Jackson Laboratory’s standard 96W chow diet (Emory Mouse, Guilford, CT) ad libitum. All mice were maintained under a strict light cycle (lights on from 0600 to 1800 h). The criteria used to distinguish neonatal fld/fld mice from the wild type and siblings were described in previous publications (Sweet et al., 1988; Langner et al., 1989) and summarized under “Results.”

Light and Electron Microscopic Studies of Brain and Sciatic Nerves—Four to 365-day-old fld/fld mice and their phenotypically normal +/? littermates were anesthetized with Avertin (0.25 mg/g of body weight) (Jones and Krohn, 1960). Following transcardiac perfusion with 0.25 × and 0.5 × Karnovsky’s fixative (diluted with 0.1 M sodium cacodylate, pH 7.4), the brain and both sciatic nerves were dissected from each animal and stored in 0.5 × Karnovsky’s fixative for 24–48 h (Karnovsky, 1965). These tissues were then transferred to 0.1 M sodium cacodylate, pH 7.4, and subsequently embedded. Brain tissue was dehydrated through a series of graded alcohol solutions and xylene and embedded in paraffin. Eight-micrometer-thick sections of paraffin-embedded brain were stained using hematoxylin and eosin, Bielschowsky’s silver, and Luxol fast blue/periodic acid-Schiff stains. Sciatic nerves were postfixed in phosphate-buffered 2% osmium tetroxide containing 1.5% potassium ferricyanide, dehydrated in graded ethanol, and embedded in Spurr’s medium with propylene oxide as an intermediary solvent. One-micrometer-thick plastic sections were stained with toluidine blue and examined by light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips 200 electron microscope.

**Western Blot Analysis of Sciatic Nerve and Brain Proteins**—Sciatic nerves or brains were pooled from 1–2, 2–3, and 3–6-month-old (termed 3+ in the text) fld/fld or +/? mice. All groups consisted of 12–24 mice, and both sciatic nerves and brains were dissected from between the 0.5 M sodium cacodylate, pH 7.4, and subsequently embedded. Brain tissue was dehydrated through a series of graded alcohol solutions and xylene and embedded in paraffin. Eight-micrometer-thick sections of paraffin-embedded brain were stained using hematoxylin and eosin, Bielschowsky’s silver, and Luxol fast blue/periodic acid-Schiff stains. Sciatic nerves were postfixed in phosphate-buffered 2% osmium tetroxide containing 1.5% potassium ferricyanide, dehydrated in graded ethanol, and embedded in Spurr’s medium with propylene oxide as an intermediary solvent. One-micrometer-thick plastic sections were stained with toluidine blue and examined by light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips 200 electron microscope.

**Isolation and Analysis of Sciatic Nerve and Brain Lipids**—Total lipids were extracted from the same pooled tissue homogenates used for the protein analyses described above. Brain and sciatic nerves (n = 16 animals, two nerves/mouse) from 6-week-old BALB/c mice (similar to the BALB/cByJ genetic background upon which the fld mutation spontaneously arose and has been subsequently maintained) were also analyzed. Homogenates containing equal protein concentrations of total protein were extracted with chloroform, methanol, 0.86% potassium chloride, 0.1 M potassium citrate (2:1:3, 6:0:6) and centrifuged at 5,000 × g for 5 min at room temperature to separate the phases. The lower phase was re-extracted with chloroform:methanol:water (3:4:8:7) (Folch et al., 1957; Webster and Folch, 1961). Phospholipid...
Phosphorus was measured using a modification of the Ames assay and used to estimate the lipid concentration of each sample extract (Duck-Chong, 1979).

Phospholipids were examined using two separation schemes. For qualitative analyses, lipids prepared from the brains and sciatic nerves of 3+–month-old BALB/c, f/d/f/d, and +/+ littermates were spotted on the lower right corners of Silica Gel H plates (14 × 14 cm, Analtech, Inc., Newark, DE). The amount of phosphatidylethanolamine applied to all plates was similar (phosphatidylethanolamine concentrations of 3+-month-old BALB/c, qualitative analyses, lipids prepared from the brains and sciatic nerves this species in brain and peripheral nerve) (Norton and Cammer, Inc., Newark, DE). The amount of phosphatidylethanolamine applied in total lipid extracts were calculated based on mole percentages of and used to estimate the lipid concentration of each sample extract.

Two-dimensional chromatography was performed using a chloroform: methanol, 7% ammonium hydroxide (120:70:9) solvent system in the first dimension and a chloroform:methanol:water (144:56:9) solvent system in the second dimension (Smith, 1969). Lipids were visualized after spraying the TLC plates with 50% sulfuric acid and charring (Marsh and Weinstein, 1966). The migration properties of phospholipid standards (Sigma) run on identical but parallel plates enabled us to identify separated spots derived from the unknown samples.

A second method was used for quantitative analysis of the individual phospholipid species present in nerve tissue. Two, 4, and 6 μg of total phospholipid from both the 1–2- and 3+-month-old f/d/f/d and +/+ sciatic nerve lipid extracts were spotted adjacent to 0.1-10 pg of phosphatidylethanolamine (Avanti Polar Lipids, Inc., Birmingham, AL) standards in the preconcentrating zone of 10 × 10-cm high performance thin-layer chromatography (HPTLC) Silica Gel 60 plates (Merck). Samples were run to the top of the plate using a petroleum ether:diethyl ether:acetic acid (90:10:1) solvent system (Mangold and Malins, 1960). Free and esterified cholesteroles were quantitated by laser densitometry of charred HPTLC plates. Briefly, samples containing 1, 2, and 4 μg of total phospholipid were spotted on plates together with cholesterol (Steraloids, Inc., Wilton, NH) and cholesterol ester standards (0.02–2 μg). Data analysis was performed exactly as described above for measurement of phospholipid levels. Actual concentrations for cholesterol and cholesterol esters were calculated after reference to the standard curves generated from each plate. Relative concentrations of triglycerides and free fatty acids were expressed as a ratio of densitometric units with respect to cholesterol for each sample (see "Results").

Neutral lipid species were analyzed in 1–2- and 3+-month-old f/d/f/d and +/+ sciatic nerve lipid extracts using a petroleum ether:diethyl ether:acetic acid (90:10:1) solvent system (Mangold and Malins, 1960). Free and esterified cholesterol levels were quantitated by laser densitometry of charred HPTLC plates. Briefly, samples containing 1, 2, and 4 μg of total phospholipid were spotted on plates together with cholesterol (Steraloids, Inc., Wilton, NH) and cholesterol ester standards (0.02–2 μg). Data analysis was performed exactly as described above for measurement of phospholipid levels. Actual concentrations for cholesterol and cholesterol esters were calculated after reference to the standard curves generated from each plate. Relative concentrations of triglycerides and free fatty acids were expressed as a ratio of their densitometric units with respect to cholesterol for each sample.

RESULTS

General Description of Neurological Symptoms in f/d/f/d Mice—Although f/d/f/d mice are identifiable as early as postnatal day 3 by their swollen abdomens and large pale livers, neurological symptoms are not overtly recognizable until postnatal day 10. They appear as a generalized tremor and unsteady gait, which progress slowly throughout life. When held up by their tails, homozygotes characteristically clench the toes of their rear feet and clasp their hind legs together (Fig. 1). Many homozygotes die between postnatal days 19 and 35. Approximately 50% of the surviving female f/d/f/d mice are fertile, but they generally do not produce their first litter until 90 days of age. Male homozygotes appear to be infertile, although this may be due to a behavioral impairment. All f/d/f/d mice have an unkempt and ruffled appearance due to reductions in hair growth.

Microscopic Analysis of Sciatic Nerves Harvested from Neonatal and Young Adult f/d/f/d Mice and Their +/+ Littermates—Sciatic nerves and brains were collected from perfusion-fixed f/d/f/d mice and their phenotypically normal +/+ littermates at 4 days and 1 month of age. Arrows indicate myelin debris (d) and enlarged Schwann cell mitochondria (mt) and nuclei (n). Magnifications: upper panels, × 4900; lower panels, × 2750.

FIG. 1. Response of f/d/f/d and +/+ mice to handling. The generalized tremor and unsteady gait in f/d/f/d mice do not become apparent until the end of the second postnatal week, at which time mutant animals can also be identified by their abnormal reaction to being picked up by the tail; the f/d/f/d mouse clenches the toes of its rear feet and attempts to clasp its hind legs together (illustrated here in the adult animals).

Fig. 2. Electron micrographs of sciatic nerves from 4-day- and 1-month-old f/d/f/d and +/+ mice. Transverse sections of f/d/f/d sciatic nerves reveal abnormally myelinated in f/d/f/d mice compared to their +/+ littermates at 4 days and 1 month of age. Arrows indicate myelin debris (d) and enlarged Schwann cell mitochondria (mt) and nuclei (n). Magnifications: upper panels, × 4900; lower panels, × 2750.

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Transmission electron microscopy showed that sciatic nerves from 4-day-old fld/fld mice had numerous axons surrounded by thin, poorly compacted myelin sheaths (Fig. 2). Occasional Schwann cells contained myelin debris. Hypertrophic Schwann cells with enlarged nuclei and abnormally large mitochondria were also observed. The age-matched +/− sciatic nerves showed no pathological changes. Electron microscopy showed that 30-, 60-, and 365-day-old +/− sciatic nerves had the expected age-dependent increase in myelin thickness (Fig. 2). Large and small myelinated axons and unmyelinated axons were readily identifiable. A number of abnormalities were detected in age-matched fld/fld mice. There were major alterations in the myelinated axon population. Numerous axons were surrounded by thin, poorly compacted myelin sheaths, and normal myelinated axons were rarely observed. Many Schwann cells appeared to accumulate myelin debris with increasing age. Hypertrophic Schwann cells were more frequent in the older fld/fld mice. In adult fld/fld mice, occasional degenerating axons, bands of Bugner, and regenerative clusters were observed. Free Schwann cell processes and basal lamina were frequently found, but onion bulb formation was rare (data not shown). No lymphocytes and only occasional macrophages were evident in the nerves. Overall, these electron microscopic abnormalities suggest a dysfunction in both the development and maintenance of myelin, i.e., fld/fld peripheral nerves show both dysmyelination and concomitant demyelination.

Western Blot Analyses of Sciatic Nerve Proteins—We analyzed myelin-associated proteins to better define the apparent PNS-specific lesion in homozygous fld mice. PNS myelin in mice contains: (i) PO (28 kDa), its main structural glycoprotein; (ii) P2 (18.5 kDa) and P3 (14 kDa), which are identical to two of the CNS myelin basic proteins; (iii) P3 (14 kDa); and (iv) myelin-associated glycoprotein (100 kDa) (Lees and Brostoff, 1984). In the CNS, proteolipid protein and the 18.5-kDa myelin basic protein serve the same structural role as the PNS-specific PO protein. Together they compose 60–80% of total CNS myelin proteins. Myelin-associated glycoprotein is also found in CNS myelin along with other minor proteins. PO and P2 are not present in the CNS (Norton and Cammer, 1984).

SDS-polyacrylamide gel electrophoresis of total sciatic nerve and brain homogenates prepared from 1–3-month-old fld/fld mice and their +/− littermates failed to reveal any differences in brain-associated proteins, but did disclose obvious changes in the steady-state levels of sciatic nerve-associated proteins (compare the left and right panels in Fig. 3A). Major reductions in the levels of 28-, 18-, and 16-kDa proteins were apparent as well as some less dramatic changes in the levels of expression of other polypeptides. Note that the concentration of a 66-kDa polypeptide was identical in fld/fld and +/− homogenates, providing an internal marker that suggests that these other changes are specific rather than part of a general reduction in the levels of all proteins.

Western blot analysis of the same tissue homogenates (see Fig. 3B) for representative Western blots at 2–3 months of age indicated that the concentration of PO was 7–13-fold less in fld/fld compared to +/− nerves at all ages surveyed. Remarkably, the P2 protein was not detectable in homozygotes sampled from 1 to 3+ months of age (Fig. 3B). P2 is a member of a family of small 14–16-kDa cytoplasmic lipid-binding proteins that includes the adipocyte-specific aP2 protein; liver, heart, and intestinal fatty acid-binding proteins (FABPs); and several proteins that bind retinoids. All are thought to participate in the uptake and/or intracellular targeting of their lipid ligands to sites of metabolic processing (e.g., Levin et al., 1988; Cistola et al., 1988; Waggoner and Bernlohr, 1998; Wootan and Storch and Bass, 1990; Li et al., 1991). The rate of synthesis of very long chain fatty acids unique to myelin correlates well with P2 mRNA levels in sciatic nerve as well as myelin formation during development (Tennekoon et al., 1980; Narayanan et al., 1988). P2 is distributed throughout the cytoplasm of Schwann cells as soon as they establish one-to-one relationship with an axon. It is also located in the cytoplasm of compact myelin (Trapp et al., 1984). These observations are consistent with the hypothesis that P2 plays a role in fatty acid elongation or in the transport of very long chain fatty acids to myelin (Narayanan et al., 1988).2

2 It is interesting that during early neonatal development, the fld mutation affects both adipocytes and hepatocytes (Langner et al., 1989), where the unlinked but homologous aP2, liver FABP (Faatp), and intestinal FABP (Fapti) genes are expressed (e.g. Hunt et al.,

FIG. 3. Determination of total sciatic nerve and brain proteins and Western blot analysis of myelin- or axon-specific sciatic nerve proteins from 2–3-month-old fld/fld and +/− tissue homogenates. Five micrograms of protein from either pooled sciatic nerve or brain homogenates (n = 18–22 animals, two nerves/animal) from fld/fld or +/− mice were reduced, denatured, and subjected to electrophoresis through 12.5% polyacrylamide gels containing SDS (0.1%) A, Coomassie Blue-stained gels showing total protein profiles; B and C, gels with sciatic nerve proteins electrophoretically transferred to nitrocellulose. Blots were probed with antibodies directed against PO, P2, and apoE (B) and neurofilament 68K and GAP-43 (C). Antigen-antibody complexes were visualized with 125I-protein A or by using a second antibody alkaline phosphatase conjugate prior to development with alkaline phosphatase substrate solution (neurofilament 68K). Arrows indicate the location of the specific protein. Note that whereas only total protein profiles and Western blot data from the 2–3-month-old animals are illustrated here, identical findings were obtained for 1–2- and 3+ -month-old animals.
Increases in apoE synthesis and secretion caused by injury to the PNS have been well documented (Skene and Shooter, 1983; Müller et al., 1985; Ignatius et al., 1986; Snipes et al., 1986). Fig. 3B shows that apoE concentrations were vastly elevated in mutant nerve homogenates. By contrast, no differences in brain apoE levels were observed between fld/fld mice and their age-matched +/? littermates (data not shown).

Two axon-specific antibody probes were incubated with our Western blots to examine whether the alterations in PNS proteins associated with the fld mutation were due to axonal degradation and concomitant degeneration of the ensheathing myelin. Neurofilament 68K is a neuron-specific subclass of intermediate filaments (Osborn and Weber, 1982; Lazarides, 1982). No differences in the steady-state levels of this protein were noted in 1-3+ month-old brain homogenates prepared from fld/fld and +/? littermates (data not shown). The concentration of the intact 68-kDa protein was somewhat reduced in fld/fld sciatic nerves at all ages surveyed, but there were elevated amounts of lower molecular mass immunoreactive species, raising the possibility of increased breakdown of this polypeptide in the PNS of homozygous animals (see Fig. 3C).

The GAP-43 protein is induced during development and axonal regeneration (Skene and Willard, 1981a, 1981b). Previous studies have suggested that GAP-43 is found only in neurons and not in Schwann cells (Meiri et al., 1988) (although one report suggested an association with the plasma membrane of rat astrocytes (Vitkovic et al., 1988). Expression of this protein was at least 3-fold greater in fld/fld sciatic nerve homogenates than in age-matched phenotypically normal +/? littermates (see Fig. 3C). Thus, our analysis of GAP-43 and neurofilament 68K expression as well as our electron microscopy studies indicated that the axons in peripheral nerves are affected by the fld mutation. This may be a secondary effect to the Schwann cells’ inability to maintain a normal myelin sheath.

An additional, higher molecular mass band also reacted by the GAP-43 antibody, but not premimmune sera (see Fig. 3C). Its intensity could be correlated with the intensity of the protein migrating at 43 kDa in fld/fld and +/? sciatic nerve homogenates. Brain homogenates contained only the expected 43-kDa species, and no differences were noted between its levels in homozygous mice and their phenotypically normal littermates. The origins and significance of the larger molecular mass species present in sciatic nerve are not known. Such a species has not been found when the same antibody was used to probe Western blots of other tissues (e.g. rat superior cervical ganglion, celiac ganglia, or postnatal day 1 brain).5

Characterization of Myelin-associated Lipids in fld/fld Mice and +/? Littermates—Total lipids were extracted from the same 1–2- and 3+-month-old fld/fld and +/? sciatic nerve and brain sciatic homogenates we used for the protein studies. Additional analyses were performed on lipids extracted from

5 S. Spencer and M. Willard, personal communication.
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BALB/c nerve and brain homogenates (fld occurred spontaneously in 1981 in the closely related BALB/cByJ mouse strain). Individual lipid species were separated by TLC using several solvent systems. Fig. 4 shows a two-dimensional separation of phospholipids present in 3+-month-old brain and sciatic nerve lipid extracts (BALB/c mice were 1.5 months old). There were no apparent differences between the representative fld/fld, +/-, and BALB/c brain homogenates (note that the phosphate content of each sample was measured prior to analysis and was used to determine that equal amounts of lipid were loaded onto the TLC plate). By contrast, sciatic nerve lipids from the fld homozygote showed an elevation of some neutral lipid species and an alteration in the relative amounts of phosphatidylserine, phosphatidyl- or phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin. The dramatic elevation in phosphatidylethanolamine levels is interesting because, as noted above, during development or regeneration, the relative amounts of this lipid species and phosphatidylethanolamine decrease from levels found in immature nerves, whereas galactosphingolipids, phosphatidalethanolamine, sphingomyelin, and cholesterol levels increase. Thus, the lipid profile of fld/fld sciatic nerves resembles that of an immature nerve or one that is in a state of repair.

The individual phospholipid species in sciatic nerve lipid extracts were quantitated after one-dimensional separation on HPTLC plates and subsequent densitometry of the charred lipid extracts. Fig. 5A shows a representative plate. Fig. 5B presents the data expressed as a ratio of the amount of a given phospholipid species to phosphatidylcholine. Fig. 5C describes the absolute amounts of phosphatidylcholine, phosphatidyl- and phosphatidylethanolamines and phosphatidylserine in 1- 2- and 3+-month-old fld/fld and +/- sciatic nerves. Fig. 5C demonstrates that: (i) phosphatidylethanolamine and its plasmalogen form are the most abundant of the three lipid species (except in 3+-month-old fld/fld mice); (ii) although there was no significant difference between phosphatidylcholine levels in the fld/fld and +/- nerves at 1-2 months, the concentration of this lipid in homozygous fld sciatic lipid extracts increased to 3.3 times the level measured in +/- nerve lipid at 3+ months of age; and (iii) the concentrations of phosphatidyl-
and phosphatidylethanolamines and phosphatidylserine are lower in fld/fld than in +/+ nerves at all ages surveyed, and these differences between homozygous mice and their +/+ littermates become larger with increasing age (see Fig. 5, B and C).

In addition to phosphatidylserine and phosphatidyl- and phosphatidalethanolamines, sphingomyelin as well as galactosphingolipids (ceresbrosides (both hydroxycerebroside and non-hydroxy cerebroside) and sulfatides (hydroxysul fatides and non-hydroxysulfatides) exhibited age-dependent progressive decreases in their levels relative to phosphatidylocholine in fld/fld but not +/+ mice (Fig. 5, A and B). Phosphatidylserinol was present in low concentrations in all samples. However, the phosphatidylserinol:phosphatidylocholine ratio was actually slightly increased in fld/fld nerve extracts when compared to values obtained with sciatic nerves lipids prepared from comparably aged +/+ mice (Fig. 5, A and B). There was an additional polar lipid species of very low abundance that ran just behind the sulfatides (labeled X in Fig. 5A). It did not show any variation between fld/fld and +/+ mice at any of the ages sampled. Two other lipid species were present only in extracts prepared from fld/fld sciatic nerves: an unknown labeled a in Fig. 5A with an Rf of 0.227 and cardiolipin (CL in Fig. 5A). Their levels were too low to be accurately quantitated. (Note that the "elevation" in cardiolipin levels may be related to the mitochondrial enlargement seen in the electron microscopic studies of fld/fld sciatic nerves presented in Fig. 2) (Heape et al., 1986).

Neutral lipids were also examined in sciatic nerve extracts from 1-2- and 3+ month-old fld/fld and +/+ mice. A representative one-dimensional HPTLC plate is shown in Fig. 6A.

The most striking finding was the elevation in cholesterol ester levels in the fld/fld lipid extracts. Cholesterol ester accumulation is considered to be a good indicator of the membrane degradation and active phagocytosis of myelin associated with the process of demyelination (Yao et al., 1980). The concentrations of cholesterol esters in fld/fld nerves did not differ significantly between 1-2 and 3+ months of age (~200 ng/µg of phospholipid) (see Fig. 6B). The -fold "induction" relative to +/+ nerves could not be determined since levels of cholesterol esters in lipid extracts prepared from these phenotypically normal littermates were too low to be detected at each of the time points sampled. Nonetheless, the maintenance of high cholesterol ester concentrations suggests that the condition does not improve with age. Cholesterol levels were significantly less in fld/fld sciatic nerve lipids compared to +/+ control nerves (526 versus 806 ng/µg of phospholipid at 1-2 months and 229 versus 526 ng/µg of phospholipid at 3+ months of age) (Fig. 6, A and B). The ratio of free fatty acids to cholesterol was similar between fld/fld and +/+ extracts at 1-2 months and modestly (2-fold) higher in homozygous mice at 3+ months of age (Fig. 6C). Triglyceride levels were also "normal" at 1+2 months. The ratio of this lipid species to cholesterol was slightly lower at 3+ months in the mutant nerves (Fig. 6C).

**DISCUSSION**

In this report, we show that the fld mutation is associated with a peripheral neuropathy that persists throughout the life of the homozygous animal, unlike the serum and liver hyper-triglyceridemia, elevations in liver apoA-IV and apoC-II mRNA levels, and tissue-specific defects in lipoprotein and hepatic lipases activities that resolve during the weaning period. The abnormal morphological appearance of peripheral nerves is associated with marked decreases in the levels of two PNS-specific myelin proteins (P0 and P2), an induction of proteins likely to be involved in a regenerative response (apoE and GAP-43), and reductions in the levels of all phospholipids and glycosphingolipids as well as an elevation of cholesterol esters. This profile is consistent with a degenerative/regenerative process, perhaps due to a dysmyelination and secondary demyelination. These changes appear to be limited to the PNS: no associated changes were noted in fld/fld CNS histology or lipids.

Continued expression of a peripheral neuropathy in fld/fld mice despite resolution of the biochemical markers that charcterize the early neonatal phenotype raises the question as to whether these early markers are secondary responses to a more fundamental metabolic disturbance that persists throughout the life of the organism. The persistence of the peripheral neuropathy in the PNS is significant given the fact that it has a vast regenerative capacity, unlike the CNS. This capacity for regeneration would allow normal myelin synthesis to occur if the fld mutation produced changes in the regulation of lipid metabolism that were confined to the suckling and weaning stages of development. Thus, it appears unlikely that the persistent PNS neuropathy results solely from the inability of fld/fld mice to properly metabolize lipids during the first 4 weeks of postnatal life.

Table I compares the morphological and biochemical changes noted in sciatic nerves of fld/fld mice to PNS changes in other mutant mice with neurological abnormalities. Like the fld/fld sciatic nerves that have Schwann cell abnormalities (such as enlarged nuclei and mitochondria), the trembler (Tr) mutation is PNS-specific, affects Schwann cells, and represents a prototypic example of what is considered a primary dysmyelination followed by a demyelination (Perkins et al.,...
Peripheral Neuropathy in fld/fld Mice

Table I
Neurological mutants with pathological changes in the PNS

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene symbol</th>
<th>Chromosome*</th>
<th>Age at onset</th>
<th>Characteristics</th>
<th>Lipid profile†</th>
<th>Life span</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crinkled</td>
<td>cr</td>
<td>13</td>
<td>?</td>
<td>Affects hair growth, enhanced by copper supplementation</td>
<td>CB † 10–20% at day 20; PE, PS, PC, SM † 10–15%</td>
<td>?</td>
<td>Falconer et al. (1992)</td>
</tr>
<tr>
<td>Dystonia</td>
<td>dt</td>
<td>1</td>
<td>6–10 days</td>
<td>Degeneration and loss of sensory nerve fibers in CNS and PNS</td>
<td>Generalized loss (10–45%) at day 12; greater loss (50–60%) at day 45</td>
<td>Weaning due to starvation, dehydration, or infection</td>
<td>Duchen and Strich (1964)</td>
</tr>
<tr>
<td>muscularum</td>
<td>dy</td>
<td>10</td>
<td>?</td>
<td>Amyelinated segments of nerve roots</td>
<td>CB † 10–20%; PL, CH normal at day 64</td>
<td>Prior to weaning</td>
<td>Michelson et al. (1955)</td>
</tr>
<tr>
<td>Jittery</td>
<td>ji</td>
<td>10</td>
<td>?</td>
<td>Hypomyelination of PNS, variable reports of effect on CNS</td>
<td>CB † 10–15%; NHSF † 40%; FE, PC, SM † 15–25% at day 25</td>
<td>?</td>
<td>DeOme (1945)</td>
</tr>
<tr>
<td>Shambling</td>
<td>shm</td>
<td>11</td>
<td>2–3 weeks</td>
<td>Deposits of materials in sensory nerve fibers of dorsal roots of spinal cord</td>
<td>PE, PS, PC, CH &lt;10%; NHSF † 28%</td>
<td>Varies, can be ≥1 year</td>
<td>Meier (1967), Green (1967)</td>
</tr>
<tr>
<td>Trembler</td>
<td>Tr</td>
<td>11</td>
<td>8–10 days</td>
<td>Hypertrophic interstitial neuropathy with hypomyelination, disorder of Schwan cells</td>
<td>CB, NHSF † 80–95%; HSF † 40%; PL † 35–80% except PC † 35%; CH † 75% at day 21; CE accumulate after day 20</td>
<td>Normal?</td>
<td>Falconer (1951)</td>
</tr>
<tr>
<td>Twitcher</td>
<td>twi</td>
<td>12</td>
<td>1 month</td>
<td>Demyelination of PNS and CNS and infiltration of macrophages with inclusion, model for Krabbe's disease</td>
<td>Uniform loss (35–45%) at day 25 in sciatic nerve</td>
<td>3 months</td>
<td>Duchen et al. (1980)</td>
</tr>
<tr>
<td>Wobblcr</td>
<td>wr</td>
<td>?</td>
<td>1 month</td>
<td>Vacuolar degeneration of motor nerve cells in brain stem and spinal cord</td>
<td>Uniform loss (10–25%) of all major lipids at day 56</td>
<td>Variable, 3 months to &gt;1 year</td>
<td>Duchen et al. (1968)</td>
</tr>
<tr>
<td>Vibrator</td>
<td>vb</td>
<td>11</td>
<td>10–12 days</td>
<td>Progressive degeneration of regionally selected neurons in CNS and PNS</td>
<td>HCB, CH † 10%; 15–40% † of all other major lipids; HSF normal</td>
<td>Dependent upon background, 1–6 months</td>
<td>Weimar et al. (1982)</td>
</tr>
</tbody>
</table>

*: Chromosome locations were taken from Peters (1990).
†: Lipid profiles were taken from Ganser et al. (1988a, 1988b).
CB, cerebrosides; PE, phosphatidyl- and phosphatidylethanolamines; PS, phosphatidylserine; PC, phosphatidylycholine; SM, sphingomyelins; PL, phospholipids; CH, cholesterol; NHSF, non-hydroxysulfatides; HSF, hydroxysulfatides; HCB, hydroxy cerebroside; CE, cholesterol esters.

1981; Heape et al., 1986). In trembler mice, Schwann cell proliferation is increased as much as 10-fold (Perkins et al., 1981). Myelin sheaths are reduced, poorly compacted, and exhibit well-developed onion bulb formations (believed to represent the results of repeated episodes of demyelination and remyelination in the same axons) (Ayers and Anderson, 1973; Aguayo et al., 1979; Low, 1976a, 1976b). As in fld/fld sciatic nerves, myelin breakdown products are evident (Ayers and Anderson, 1973). In trembler sciatic nerves, myelin breakdown products are evident (they are reduced 80–95%, 55–80%, and 75%, respectively, by postnatal week 3 in mice that are homozygous for the Tr mutation (see Table I). With the exception of cardiolipin, lipid accumulation ceases in the trembler PNS after postnatal day 18, leading to a worsening of the lipid deficiency in peripheral nerves as these homoygous mice age (Heape et al., 1986). Cholesterol esters, indicators of membrane degradation and markers for demyelination, are detected after 18 days (they are reduced 29–71% and 67–88% at 1–2 and 3+ months, respectively) (see Fig. 5B). As in trembler mice, phosphatidylcholine is least affected, whereas phosphatidylserine is most affected (Larrouqué-Régnier et al., 1979). Triglyceride and cholesterol levels are also reduced, although not as dramatically as in...
trembler sciatic nerves. The relative increase of cholesterol esters strongly suggests that the \( \text{fld}/\text{fld} \) sciatic nerve undergoes demyelination/remyelination. This notion is supported by the tissue-specific changes in apoE (elevated in the PNS, but not the CNS) in the peripheral nerves of \( \text{fld} \) homozygotes (see Fig. 3B and Langner et al., 1989). ApoE is produced in the PNS by nonmyelinating Schwann cells and infiltrating nonresident macrophages (Boyles et al., 1985; Stoll and Mueller, 1986). As noted above, it is thought to play a role in the scavenging of released cholesterol, which permits its storage and reutilization during nerve regeneration (Boyles et al., 1989).

We can only speculate as to the significance of the similarities between \( \text{fld}/\text{fld} \) and trembler mice. There are many other mouse mutations with significant pathological changes involving the PNS (summarized in Table I and by Gams et al., 1988a, 1988b). These include twitcher (\( \text{twi} \)), dystrophia muscularis (\( \text{dy} \)), wobbler, (\( \text{wr} \)), jittery (\( \text{ji} \)), vibrator (\( \text{vb} \)), and dystonia musculorum (\( \text{dt} \)). These mutations, with the exception of \( \text{twi} \), result in much less dramatic alterations in PNS lipids (10-40% reductions) compared to \( \text{Tr} \) or \( \text{fld} \). Interestingly, none of the mice with these mutations have detectable levels of cholesterol esters in any of the peripheral nerves examined. Given the wide variation in themes for myelin lipid and protein changes in known mutants with neurological deficits, it would be difficult to conclude from the data presented here that the \( \text{fld} \) and \( \text{Tr} \) mutations affect a common metabolic pathway. The trembler mouse has a primary defect in its Schwann cells (reviewed by Bray et al., 1981). The morphological and biochemical similarities between these two mutants suggest, but certainly do not prove, that the \( \text{fld} \) mutation may have had a specific effect on some aspect of Schwann cell metabolism. Trembler mice show prominent onion bulb formations in their sciatic nerves that are not present in \( \text{fld}/\text{fld} \) mice. These animals also lack any known axonal and non-nervous system defects, as are found in the \( \text{fld} \) homozygotes (Aguyao et al., 1977; Pollard and McLeod, 1980). Analysis of aggregation chimeras generated with trembler and \( \text{fld}/\text{fld} \) embryos as well as the identification of the \( \text{fld} \) locus may provide interesting clues about functional similarities and/or differences between the \( \text{Tr} \) and \( \text{fld} \) mutations.

A number of human diseases produce neuropathies associated with altered lipid metabolism, e.g., the heritable myelin diseases of sulfatide lipidosis, galactosylceramide lipidosis (Krabbe's disease), and Refsum's disease (Raine, 1984; reviewed by Scambagh et al., 1983). Of these examples, only Refsum's disease involves a much greater lipid loss (due to extensive degeneration) in the PNS than in the CNS (MacBrinn and O'Brien, 1968). The neuropathy in this disease is somewhat reminiscent of that seen in \( \text{fld} \) and \( \text{Tr} \) mice.

\* The \( \text{fld} \) mutation has not yet been mapped. Southern blot analyses (data not shown) have provided no evidence that \( \text{fld} \) mutation was caused by a retroviral insertion (the apparent cause of \( \approx 5\% \) of all spontaneous recessive mutations in mice) (Stoye et al., 1988). Mapping studies have determined that there is no obvious linkage of \( \text{fld} \) to hemoglobin \( \alpha \) (\( \text{HbA} \)) or \( \text{beta} \) (\( \text{HbA} \)) genes located both proximal and distal to the \( \text{Tr} \) mutation on chromosome 11. Close linkage of \( \text{fld} \) to \( \text{Tr} \) has therefore been excluded. It is also unlikely that the \( \text{fld} \) mutation is linked to any of the other neurological mutations listed in Table I that have been mapped. \( \text{fld} \) is not linked to pink-eyed dilution (\( \text{pd} \)), which is close enough to apoE (\( \text{ApoE} \)) on chromosome 7 to suggest nonlinkage of \( \text{fld} \) to apoE. \( \text{Tr} \) has been assigned to chromosome 1 (Kuhn et al., 1990). Whereas its exact location is unknown, linkage analysis with many markers for chromosome 1 suggested that the \( \text{fld} \) locus does not reside on this mouse chromosome. The \( \text{fld} \) mutation has been determined not to be linked to carbonic anhydrases 1 and 2 (\( \text{Car} \)-1 and \( \text{Car} \)-2) on chromosome 1, excluding the possibility of linkage to Ap2, the gene encoding Ap2 (M. T. Davison and H. O. Sweet, personal communication).

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