Differential Neuropathies in Hyperglycemic and Hypoglycemic Diabetic Rats

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INTRODUCTION

Diabetic neuropathy is a major long-term complication in patients with diabetes (1), and it is believed that hyperglycemia plays an important role in development of this condition. It has been shown that improved glycemic control resulting in glucose and HbA1c values close to normal levels can delay the onset and slow the progression of complications in patients with diabetes (2, 3). Therefore, it has been recommended that patients with type 1 diabetes be given intensive insulin treatment to avoid hyperglycemia, although such treatment may increase the occurrence of hypoglycemic episodes (4–6). Bulsara et al (6) have observed that reduction of HbA1c from 11% to 9% raised the rate of severe hypoglycemia 3 to 4 times in patients with type 1 diabetes. Hypoglycemia may cause peripheral neuropathy as well as pathologic changes in the central nervous system (7). Indeed, the mixed picture of motor and sensory neuropathy seen in patients with diabetes may be the result of the combined effect of hyper- and hypoglycemia. Diabetic neuropathy has been the subject of numerous clinical and experimental investigations, but few studies have focused on hypoglycemic neuropathy.

Studies of the plantar nerves in insulin-treated diabetic rats have revealed that hypoglycemic neuropathy includes a distal, Wallerian-type degeneration of large myelinated axons in the peripheral nervous system (8, 9). Our previous studies done on diabetic rats also showed that hypoglycemia has a more severe impact on motor nerve terminals in hind paw muscle than on sensory nerve endings in hind paw skin (10) and that hypoglycemic neuropathy affects large myelinated axons in the L5 ventral root but does not influence similarly sized axons in the L5 dorsal root (11). These observations suggested that hypoglycemic neuropathy preferentially affects motor nerves. In support of that assumption, it was recently found that large myelinated vagus nerve axons destined to the laryngeal muscles showed obvious signs of degeneration in insulin-treated hypoglycemic diabetic rats (12).

Against this background, the aim of the present study was to assess the different effects of hyperglycemia and hypoglycemia on the development of motor and sensory neuropathy. Accordingly, we examined the lateral and the medial gastrocnemius nerves (LGN and MGN) projecting to calf muscles and also the sural nerve (SN), which is a cutaneous sensory nerve with very few motor axons (13, 14). To our knowledge, this is the first study in which the impact of long-term hyper- and hypoglycemia on a purely somatic motor nerve and a somatic sensory nerve was analyzed.

MATERIALS AND METHODS

The study protocol was approved by the local ethics committee. Homozygous prediabetic female BB/Wor rats (n = 10) and heterozygous healthy female siblings (n = 5)
were kept in standard plastic cages with soft bedding and filtered air supply. The prediabetic and nondiabetic animals had been identified by genetic analysis (15). Onset of type 1 diabetes in the prediabetic rats was signaled by weight loss and hyperglycemia (>10 mmol/L), and at that stage, the animals were randomly divided into a hyperglycemic and a hypoglycemic insulin treatment group. The rats in the hyperglycemic group (n = 5) were treated with 3- to 4-mm-long subcutaneous insulin implants (Linplant; Linshin-Canada, Ontario, Canada) inserted under the dorsal interscapular skin, and those in the hypoglycemic group (n = 5) received a whole implant (7 mm). The insulin treatment lasted 3 months (97 ± 2 days) during which time the individual rats usually had 3 or 4 successive insulin implants (8). Blood glucose was measured 3 times a week in diabetic animals and once a week in controls using a drop of blood from the tip of the tail with a Glucometer Elite device.

Animals and Insulin Treatment

Diabetic and Hypoglycemic Neuropathy

Three-month-old male BB/Wor rats were used as the experimental animals. The rats were divided into diabetic and nondiabetic groups. The diabetic group received 1.5 units of insulin daily (a total of 15 units in a 3-month period). The nondiabetic group received no insulin treatment. After 3 months of insulin treatment, the rats were perfused with Tyrode's solution, and the sciatic nerves were removed. The sciatic nerves were then divided into hypoglycemic and hyperglycemic groups. The hypoglycemic group was sampled from the left sciatic nerve, and the hyperglycemic group was sampled from the right sciatic nerve. The sciatic nerves were then fixed in glutaraldehyde, postfixed in osmium tetroxide, and dehydrated in an ethanol series. The nerves were then embedded in Epon 812, and 1-μm-thick sections were cut. These sections were stained with toluidine blue and examined microscopically.

RESULTS

Animals and Insulin Treatment

On average, rats were 66 days old (range, 51–80 days) and weighed 169 g (range, 154–204 g) at onset of manifest diabetes. After insulin treatment was initiated in the diabetic animals, the blood glucose level dropped markedly and fluctuated to some extent, especially at the beginning of the treatment period. The effects of insulin treatment on blood glucose concentration varied among the animals (Table 1) with an average level of 5.8 ± 0.5 mmol/L (median, 5.5 mmol/L) in controls, 22.0 ± 7.8 mmol/L (median, 22.3 mmol/L) in hyperglycemic rats, and 4.1 ± 3.3 mmol/L (median, 3.1 mmol/L) in hypoglycemic animals. The mean HbA1c value was significantly higher in hyperglycemic rats (8.1%; p < 0.001) than in the controls (2.9%) and the

| Table 1. Weight of Nondiabetic Control and Diabetic BB/Wor Rats at the End of the Experiment and the Number of Days of Hyperglycemic or Hypoglycemic State During 3 Months of Insulin Treatment |
|---|---|---|---|---|---|
| Animals | 1 | 2 | 3 | 4 | 5 |
| Control group | | | | | |
| Weight (g) | 244 | 222 | 239 | 281 | 263 |
| Hyperglycemic group | | | | | |
| Weight (g) | 218 | 189 | 240 | 220 | 228 |
| Days with BG ≥8 mmol/L | 74 | 90 | 96 | 103 | 65 |
| Days with BG ≥15 mmol/L | 51 | 62 | 75 | 81 | 57 |
| Hypoglycemic group | | | | | |
| Weight (g) | 225 | 253 | 275 | 244 | 225 |
| Days with BG ≤3.5 mmol/L | 49 | 47 | 46 | 31 | 63 |
| Days with BG ≤2.5 mmol/L | 27 | 22 | 22 | 10 | 38 |

BG, blood glucose.
hypoglycemic group (2.5%). At the end of the experiments, the average hyperglycemic rat weighed significantly less ($p < 0.05$) than the control and hypoglycemic animals. Two rats in the hypoglycemic group (Table 1) that had the lowest glucose profile during the treatment period developed a characteristic grunting sound related to partial denervation of laryngeal muscles (12).

**Gastrocnemius Nerves**

**Hyperglycemic Rats**

The preparation of teased LGN from hyperglycemic rats was similar to the controls (Fig. 1A, B; Table 2). There were no signs of degeneration and regeneration or demyelination of myelinated fibers. Electron microscopy indicated that the MGN samples displayed a qualitatively normal pattern (Fig. 2A, B). The peak in diameter of myelinated fibers was shifted slightly to the left (Fig. 3A, B). The number of myelinated and unmyelinated fibers and the diameter, occupancy, density, and $g$-ratio of myelinated fibers were normal (Table 2).

**Hypoglycemic Rats**

Teased LGN from all hypoglycemic rats showed obvious signs of past and ongoing degeneration of large myelinated nerve fibers. Many larger myelin sheaths were wrinkled or had disintegrated into large ovoids or rows of smaller ovoids and clusters of myelin debris. There was no evidence of paranodal and segmental demyelination or intercalated nodes. Internodal length and diameter measurements indicated that internodes in LGN were shorter and thinner in hypoglycemic rats than in control and hyperglycemic animals (Fig. 1A–C, Table 2). The internodes were composed of 2 separate populations (Fig. 1C). In one of the populations, the length-to-diameter relationship resembled the normal pattern in that the lengths increased from approximately 200 to around 1100 $\mu$m and the diameters increased from 2 to approximately 15 $\mu$m. The nerve fibers in this group of animals had fewer large internodes compared with the control and hyperglycemic rats. The other population of internodes maintained lengths of approximately 200 to 300 $\mu$m, whereas diameter varied between 2 and 8 $\mu$m (Fig. 1C).

**TABLE 2. Morphometric Data on Lateral and Medial Gastrocnemius Nerves (LGN and MGN) of Nondiabetic Control Rats and Hyperglycemic and Hypoglycemic Insulin-Treated Diabetic Rats**

<table>
<thead>
<tr>
<th></th>
<th>LGN (LM)</th>
<th></th>
<th></th>
<th>MGN (EM)</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L ($\mu$m)</td>
<td>D ($\mu$m)</td>
<td>MDP</td>
<td>MF</td>
<td>UMF</td>
<td>D ($\mu$m)</td>
</tr>
<tr>
<td>Control (n = 5)</td>
<td>708.7 ± 45.6</td>
<td>8.3 ± 0.6</td>
<td>257 ± 7</td>
<td>472 ± 46</td>
<td>7.0 ± 0.6</td>
<td>0.62</td>
</tr>
<tr>
<td>Diabetic hyperglycemic (n = 5)</td>
<td>702.6 ± 61.7</td>
<td>8.4 ± 0.6</td>
<td>277 ± 71 (39%)</td>
<td>427 ± 77</td>
<td>6.3 ± 0.4</td>
<td>0.64</td>
</tr>
<tr>
<td>Diabetic hypoglycemic (n = 5)</td>
<td>496.5 ± 136.6 (p = 0.01)*</td>
<td>6.5 ± 1.1 (p = 0.01)*</td>
<td>8</td>
<td>255 ± 87 (p = 0.02)b</td>
<td>436 ± 97 (p = 0.03)*</td>
<td>5.4 ± 1.2 (p = 0.03)*</td>
</tr>
</tbody>
</table>

Diabetic animals were treated with insulin implants for 3 months.
LM, light microscopy; EM, electron microscopy; L, internodal length; D, fiber diameter including myelin sheath; MDP, mean number of myelin degradation products seen in nerve cross-sections; MF, no. of myelinated fibers; UMF, no. of unmyelinated fibers; $g$-ratio, axon/fiber diameter ratio; %, percentage of total number of fibers; p, significant difference compared with *, control and †, hyperglycemic rats. Data are presented as mean ± standard deviation.
Ultrastructural examination of MGN from hypoglycemic rats showed that the occurrence of large and medium-sized myelinated axons was markedly reduced compared with controls (Fig. 2A, C). We observed a variety of pathologic features, including myelin degradation products, empty Schwann cells, collagen pockets, Büngner bands, and regeneration unit-like structures (Fig. 4A–C) as well as few relatively large axons (diameter approximately 2 μm) with very thin myelin sheaths (Fig. 4D). All hypoglycemic animals exhibited these characteristics, although the frequency varied between individual rats. Qualitative assessment indicated that presence of the mentioned pathologic features was positively related to the duration of severe hypoglycemia in these rats. Considering unmyelinated nerve fibers in MGN samples, the picture was similar to controls. Fiber counting showed that the average MGN contained 691 axons, 37% of which were myelinated. The mean diameter of the fibers was 5.4 μm, which was significantly smaller than the corresponding values for the controls (p = 0.03; Table 2), and the size distribution was markedly shifted to the left (Fig. 3A, C). In addition, there was a strong correlation (r = −0.9) between duration of severe hypoglycemia (≤2.5 mmol/L) and the mean diameter (Fig. 5A). The mean g-ratio was normal. Occupancy, but not density, of myelinated fibers was reduced compared with controls, which implies that small regenerated fibers had replaced large myelinated fibers. Both density and occupancy were reduced in the hypoglycemic rats compared with the hyperglycemic group (Table 2), which probably reflects the smaller endoneurial space in the hyperglycemic animals.

Sural Nerve

Hyperglycemic Rats

Teased myelinated SN fibers from hyperglycemic rats were qualitatively similar to controls. There were no signs of
nerve fiber pathology and both internodal length (not significant) and diameter \((p = 0.008)\) measurements were smaller compared with controls (Fig. 1D, E; Table 3). The internodal length increased from approximately 200 to around 800 \(\mu m\), and the diameter increased from 2 to approximately 9 \(\mu m\) (Fig. 1E). Analysis of SN by electron microscopy indicated that the nerve fibers and other elements such as perineurium, endoneurial connective tissue, and blood vessels were similar to controls. There were no signs of degenerated or demyelinated nerve fibers (Fig. 2D, E). On average, there were a total of 3,250 fibers, in which 23% were myelinated. The myelinated fibers in these rats were significantly smaller and the size distribution histogram was shifted to the left compared with controls (Fig. 3D, E; Table 3). The decreased mean fiber diameter in individual animals was correlated \((r = 0.8)\) with decreased body weight (Fig. 5B). The mean \(g\)-ratio was similar to controls. Myelinated fiber occupancy was the same as seen in controls, whereas fiber density was increased (Table 3).

### Hypoglycemic Rats

Teased samples of SN from hypoglycemic rats were slightly wrinkled and a few large myelinated fibers showed signs of past or ongoing degeneration. There were no qualitative features suggesting axonal demyelination or remyelination. Compared with the controls, both length and diameter measurements were smaller as a result of absence of the largest fibers (Fig. 1D, F; Table 3). Lengths increased from 200 to 800 \(\mu m\), and diameters increased from 2 to approximately 9 \(\mu m\) (Fig. 1F). Electron micrographs...

![Graphs](image1.png)

**FIGURE 3.** Histograms illustrating the distribution of diameters (D, myelin sheath included) of myelinated fibers in the (A–C) medial gastrocnemius nerve and the (D–F) sural nerve of all rats in the (A, D) nondiabetic control group, the (B, E) diabetic hyperglycemic group, and the (C, F) diabetic hypoglycemic group. \(n\), number of observations.

![Micrographs](image2.png)

**FIGURE 4.** Electron micrographs showing different pathologic findings in the medial gastrocnemius nerve of a diabetic rat treated with insulin implants to maintain a hypoglycemic state for approximately 3 months. (A) Myelin degradation products (thick arrows) and an intracellular myelin-like structure (thin arrow). Original magnification: 1,500×. (B) Early phase of axonal regeneration seen as a Büngner band. Note the limiting basal membrane. Original magnification: 10,000×. (C) Collagen pocket. Original magnification: 30,000×. (D) A relatively large axon with a thin myelin sheath. Original magnification: 6,000×. Nerve preparations were treated with osmium tetroxide and contrasted with uranyl acetate and lead citrate.
indicated that SN samples from hypoglycemic rats were essentially comparable to SN in control and hyperglycemic rats (Fig. 2D–F), although they did contain some isolated myelin debris and a few Schwann cells that lacked associated axons but possessed collagen pockets. No other abnormalities were observed in endoneurial or perineurial nerve elements. SN had markedly fewer pathologic features than MGN did (Tables 2 and 3). There were no obvious alterations in the morphology of unmyelinated fibers in SN. Counting indicated that the average SN in hypoglycemic rats contained 3,875 axons, 20% of which were myelinated (Table 3). The mean diameter in these rats was significantly decreased and showed a unimodal distribution pattern with a distinct reduction in the number of large myelinated fibers (Fig. 3F). The mean g-ratio was normal. The pattern of myelinated fiber occupancy and density was the same as in MGN in these animals. In other words, the hypoglycemic rats displayed a significant decrease in occupancy compared with controls, whereas they showed a drop in both occupancy and density compared with hyperglycemic rats (Table 3).

### TABLE 3. Quantitative Assessments of the Sural Nerve of Nondiabetic Control Rats and Hyperglycemic and Hypoglycemic Insulin-Treated Diabetic Rats

<table>
<thead>
<tr>
<th></th>
<th>LM</th>
<th>EM</th>
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<tbody>
<tr>
<td></td>
<td>L (μm)</td>
<td>D (μm)</td>
</tr>
<tr>
<td>Control</td>
<td>602.8 ± 48.8</td>
<td>6.3 ± 0.6</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
<td>(23%)</td>
</tr>
<tr>
<td>Diabetic hyperglycemic</td>
<td>521.6 ± 76.0</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>(p = 0.005)*</td>
<td>(23%)</td>
</tr>
<tr>
<td>Diabetic hypoglycemic</td>
<td>495.8 ± 41.2</td>
<td>5.4 ± 0.3</td>
</tr>
<tr>
<td>(n = 5)</td>
<td>(p = 0.006)*</td>
<td>(20%)</td>
</tr>
</tbody>
</table>

The diabetic animals were treated with insulin implants for 3 months. LM, light microscopy; EM, electron microscopy; L, internodal length; D, fiber diameter including myelin sheath; MDP, mean number of myelin degradation products seen in nerve cross-sections; MF, no. of myelinated fibers; UMF, no. of unmyelinated fibers; g-ratio, axon/fiber diameter ratio; %, percentage of total no. of fibers; p, significant difference compared with †, control and ‡, hyperglycemic rats. Data are presented as mean ± standard deviation.

**FIGURE 5.** (A) Correlation between the duration of severe hypoglycemia (<2.5 mmol/L) and the mean diameter (D) of myelinated fibers of gastrocnemius nerve in the hypoglycemic group (r = −0.9). (B) Correlation between body weight of the hyperglycemic rats at the end of the experiment and the mean diameter (D) of myelinated fibers of the sural nerve (r = 0.8).

**DISCUSSION**

In this study, we compared the neuropathologic impact of prolonged hyper- and hypoglycemia on 2 purely muscle-related nerves and a skin-related peripheral nerve in type 1 diabetic BB/Wor rats. The results revealed that prolonged hyperglycemia affects the somatic sensory fibers by decreasing the diameter of large myelinated fibers without causing axonal degeneration or demyelination. Prolonged hypoglycemia, on the other hand, preferentially and more severely influences the somatic motor fibers, leading to Wallerian-like axonal degeneration and regeneration of large myelinated nerve fibers. However, the functional significance of these morphologic alterations is not yet known.

According to our nerve fiber counts, control MGN contained 729 axons, 35% of which were myelinated, which agrees well with results obtained by other investigators (16, 17). However, our control SN contained fewer axons than reported by other researchers (18–20). This discrepancy might be explained by differences in sex and the rat models used in the various studies. The female BB/Wor rats in our study weighed less than the male SD rats used in most investigations. Moreover, it is known that the number of axons in the SN varies considerably between different sides of the body and between individual rats (21, 22). Different counting methods may also have contributed to the disparate findings. The bimodal pattern and the size distribution of the myelinated fibers with mean diameter of approximately 7 and 5.5 μm in the MGN and SN, respectively, conform well with the results of other studies (21–25).

In our hyperglycemic animals, the mean diameter of myelinated fibers in the SN, but not the GN, was smaller compared with controls. Inasmuch as we did not observe any signs of axonal degeneration and regeneration or demyelination and remyelination in the nerve samples, the smaller diameter must have been caused by other factors. Other researchers have also found decrease diameters of fibers in the absence of axonal degeneration and regeneration in diabetic animals (26–29), and they have suggested that the reduced size might be explained by factors such as hyperosmolality, deranged axonal metabolism, or retarded growth.

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1123
Furthermore, it has been proposed that loss of neurofilaments could be involved in reduced myelinated fiber size in hyperglycemic rats (24, 26, 30). We recorded similar g-ratios for both SN and MGN and for different group of animals, which established that there was no axonal atrophy. In addition, the occupancy of myelinated fibers in the MGN and SN of the hyperglycemic rats was similar to the occupancy observed in the controls, whereas the density of myelinated fibers in the SN was significantly higher than in the controls. Our hyperglycemic animals weighed significantly less than the controls, suggesting that the smaller fiber diameter and higher myelinated fiber density in the hyperglycemic rats may be the result of retarded growth or abnormal metabolism. However, if this was the case, we should have observed smaller diameter and higher fiber density in the MGN as well. Because the SN and GN react differently to hyperglycemia, it is possible that other factors are involved in development of diabetic neuropathy. The glucose concentration is apparently higher in skin than in muscle (31). Accordingly, it is possible that the glucose levels in hyperglycemic rats are higher in skin than in muscle, which in turn leads to more pronounced axonal damage in the sensory nerve at the target organ. This raises the question of whether target organs contribute to the development of neuropathy in diabetes.

The individual diabetic rats in our study responded differently to the same insulin treatment protocol, and hence the degree and duration of hyper- or hypoglycemic periods varied between animals within the same group (12) (Table 1). In the hypoglycemic animals, the most affected nerves were seen in the rats with longest duration of severe hypoglycemia (≥2.5 mmol/L). This was confirmed with an obvious correlation (r = −0.9) between duration of severe hyperglycemia and mean myelinated fiber diameter in individual hypoglycemic rats. These observations support the idea that the duration and severity of hypoglycemia may be related to the severity of neuropathy (32, 33).

The pathologic picture in our hypoglycemic rats was dominated by degeneration of large myelinated axons. We found no evidence of demyelination and remyelination such as occurrence of short and thick internodes in teased preparations or electron micrographs showing frequent appearance of large axon profiles that lacked myelin or had only a thin myelin sheath. Diameter measurements revealed a shift to smaller fibers in the absence of axonal atrophy, as suggested by normal g-ratios. In addition, the occurrence of many small myelinated axons and regeneration unit-like structures signaled the presence of past and ongoing axonal regeneration. In the hypoglycemic MGN, only 12% of the fibers had a diameter >9 μm, which is far less than the level of almost 60% in control MGN. Almost 25% of the fibers in hypoglycemic SN had a diameter >6 μm, whereas the rate was 50% in controls. The myelinated fiber occupancy, but not fiber density, was decreased in MGN and SN. These findings suggest that degeneration preferentially affected larger fibers and that the disappearance of large myelinated axons was compensated for by the presence of many small myelinated axons. Degeneration of myelinated axons may be a consequence of hypoglycemia, whereas the increased densities of small myelinated axons might be induced by hyperinsulinemia (33). Notably, endoneurial microvascular abnormalities that other investigators have observed in rats with short-term (34) or long-term hypoglycemia (35) did not occur in our animal model. Taken together, the present results imply that hypoglycemia provoked by excessive insulin treatment in diabetic rats causes degeneration and regeneration of larger myelinated fibers that preferentially affect muscle-related nerves.

The rat MGN contains intrafusal somatic motor fibers as well as sensory and sympathetic fibers (17). The alpha motor nerve fibers to the extrafusal muscle and the Ia afferent axons are the largest myelinated fibers. Furthermore, not only does the rat SN supply sensory and sympathetic fibers to the skin, but it also innervates foot muscles through the lateral plantar nerve (13, 14, 17, 36). Thus, it is likely that the degenerated large fibers in the both the GNs and SN in this study were muscle-related. It is plausible that hypoglycemia is detrimental to nerve fibers with a large caliber, but it is also possible that it preferentially damages fibers innervating the muscle. The consequence in both cases is that skeletal muscle, but not skin, would preferentially be affected by hypoglycemia. As discussed previously, the glucose level in muscle may be lower than in skin. If so, the glucose concentration in hypoglycemia may drop to an even lower level in muscle than that in skin and thereby cause more extensive axonal damage in the former tissue. Study of glucose levels in different peripheral tissue and the role of it in development of hypoglycemic neuropathy is the objective for our future work.

The risk of hypoglycemia is the major impediment to achieving optimal glycemic control in patients with diabetes. An average patient with type 1 diabetes has a low plasma glucose level approximately 10% of the time, and 2% to 4% of the deaths in these patients are attributed to hypoglycemia (37). The clinical, electrophysiological, and morphologic features of neuropathologic complications in some patients with diabetes are similar to those noted in insulinoma patients with frequent severe hypoglycemic episodes (38). These observations suggest that the neuropathy seen in patients with diabetes may be the product of periods of alternating hyperglycemic and hypoglycemic state. It is now known that hyperglycemia is an important factor contributing to the development of diabetic neuropathy, but the major challenge is to determine the role of hypoglycemia in development of neuropathy in patients with insulin-treated diabetes.

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REFERENCES


2. Diabetes Control and Complications Trial Research Group. The effect
of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus.


17. Sittiracha T, McLachlan EM. Evaluation of the effects of various additives on retrograde labelling by horseradish peroxidase applied to the sciatic nerve on horseradish peroxidase labelling of primary sensory neurons. Brain Res 1984;299:9–14


