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Gangliosides Accelerate Rat Neonatal Learning and Levels of Cortical Acetylcholinesterases

Stephen E. Karpiak, Ferdinand Vilim, S.P. Mahadik

Division of Neuroscience, New York State Psychiatric Institute and Departments of Psychiatry and Biochemistry, College of Physicians and Surgeons, Columbia University, New York, N.Y., USA

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Abstract. Several studies have shown that exogenous gangliosides can stimulate neurite outgrowth (in vitro), accelerate peripheral nerve regeneration (in vivo) and facilitate CNS recovery after lesioning. Experiments were designed to assess the effects of ganglioside administration on neonatal development. Rat neonates received daily subcutaneous injections of gangliosides from PN day 5 through 15. Learning behavior (acquisition and retention) was facilitated in rats which had received injections of either total, GM1 or GD1b ganglioside. Rats injected with GD1a and GT1b ganglioside were not different from controls. Levels of total AChE activity as well as its 4s and 10s molecular forms in cortex, were assayed at PN days 9, 14, 21 and 28 in rats injected with GM1 ganglioside. These animals had consistently higher levels of enzyme activity as compared to saline controls. It is hypothesized that exogenous gangliosides accelerate CNS maturation.

Introduction

Gangliosides are found in high concentration in the CNS [4, 32], and because of their topographical localization to the outer plasma membrane surface of neurons [8, 9, 19], they have been implicated as receptor molecules, particularly for 'trophic growth factors' [6, 7, 18, 37]. Some hypotheses propose that gangliosides participate in the regulation of neurogenesis [38], synaptogenesis [22], regeneration [7] as well as cell-cell interaction [40].

Reports that antibody to ganglioside can inhibit neurite outgrowth in vitro [31, 33] indirectly support hypotheses which propose

that gangliosides are receptor molecules for 'trophic growth factors'. In collaborative studies [16] we have shown that perinatal exposure of rats to antibody to ganglioside results in behavioral deficits when the animals are tested at adult age. These deficits are paralleled by neurochemical alterations such as reduced levels of ganglioside sialic acid and galactocerebroside as well as alterations in morphology of cortical dendritic spines. It was hypothesized that the antibody to ganglioside interfered with dendritic formation resulting in hypomyelination and aberrant synaptogenesis [16].

Other evidence in support of a critical role for gangliosides in neuronal development is based on reports that exogenous gangliosides can accelerate neurite outgrowth in vitro [3, 18, 28] and enhance peripheral nerve regeneration after injury [7, 17]. More recent studies by us [13–15] and by other investigators [23, 30, 37] indicate that ganglioside administration may facilitate regeneration and functional recovery after CNS damage.

Mechanisms underlying these effects are not yet clarified. However, it has been demonstrated that incubation [35, 36] of rat brain neuronal membranes with GM1 ganglioside results in their insertion into the plasma membrane. These 'inserted' gangliosides are termed 'functional' since cells incubated with GM1 ganglioside show increased choleragen binding [5, 24]. The inserted GM1 ganglioside persists for as long as 4 days. NMR studies confirm this 'insertion' phenomenon [10].

Our preliminary studies have shown that learning behavior in the rat neonate PN days 10–15 was facilitated by injections of total brain gangliosides [11, 12]. The study reported here was undertaken (1) to further assess this facilitatory effect of gangliosides on the developing neonate; (2) to determine whether individual ganglioside species showed differences in effectiveness, and (3) to monitor a neurochemical parameter, namely acetylcholinesterase (total AChE activity as well as the 4s and 10s molecular forms) in cortex of treated animals. Assaying AChE and its molecular forms in the cortex seemed pertinent because changes in cholinergic activity reportedly parallel alterations in cognitive function [25, 29].

Materials and Methods

Animals

Neonates from Sprague-Dawley rats were used throughout these experiments. All rat litters were culled to 8. In any given litter only one pair of animals received identical ganglioside injections. Consequently, injections of the different ganglioside species were randomized throughout the litters. Animals were weighed daily.

Ganglioside Injections

Beginning on PN day 5 through PN 15 each neonate received a daily subcutaneous injection (in 0.025 ml sterile saline) of 2.5 mg of either total brain ganglioside (average composition: GM1 21%; GD1a 39%; GD1b 16%; GT1 19%), or one of the following ganglioside species: GM1, GD1a, GD1b or GT1b (FIDIA Research Laboratories, Abano Terme, Italy). Controls received saline.

Behavioral Testing

On PN days 11, 12 and 14, rats were trained on a multidirectional avoidance paradigm [21]. Performance was measured as the time latency to escape shock (scrambled: 0.1 mA). Rats received 5 consecutive trials with an intertrial interval of 10s. The current was terminated for any given trial if the rat did not escape after 15s. Escape was established when the neonate placed 3 limbs on to the escape platform. At least 6 rats (e.g. 2 from each of three different litters) were tested for each treatment (n=42).

Behavioral Apparatus

The testing apparatus consisted of a raised platform (8×8 cm). In the center of this platform was a well (4×4 cm) sunken 0.9 cm below the surface on the platform. At

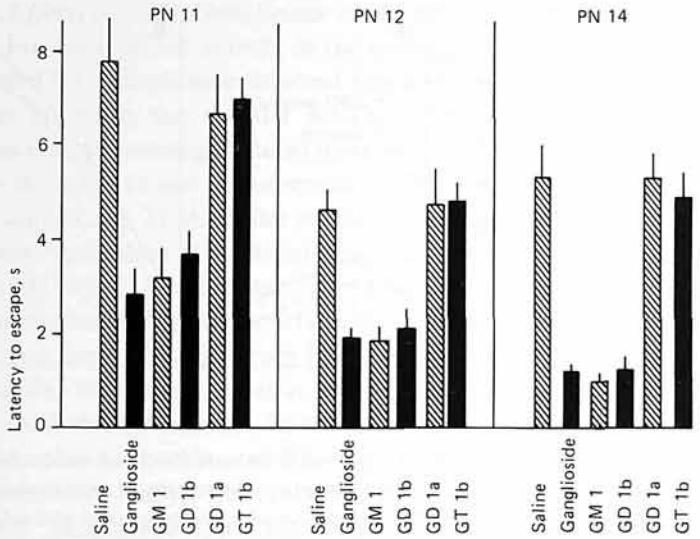

Fig. 1. Learning performance (latencies) of rat neonates treated with either total ganglioside or individual ganglioside species. Controls received saline. Facilitated acquisition (PN 11) and retention (PN 14) is seen in those animals treated with either total ganglioside, GM1 or GD1b ganglioside.

the bottom of the well were 12 stainless steel grids which were connected to a scrambled shock source (BRS No. 2903). The surrounding escape platform (ledge) was covered with a canvas cloth to allow the rats a surface which could easily be gripped in order to pull themselves from the well and avoid the shock.

AChE Analyses

Independent groups of rat pups were used for neurochemical analysis. Comparisons were made only between rats which were injected with GM1 ganglioside (daily subcutaneous injections PN days 5–15) or saline. Assay for AChE activity was done on the dissected cortices of these animals (n=3 for each time point) on PN days 7, 14, 21 and 28. Animals were randomized among eight different litters.

Acetylthiocholine, 3,3′-dithiobis[6-nitrobenzoic acid] (DTNB) and 'ISO-OMPA' were obtained from Sigma Chemicals (St. Louis, Mo.). All other reagents for buffers, and for sucrose gradients (grade A) were purchased from Fisher Scientific (Fair Lawn, N.J.).

Tissue extracts were prepared from the pooled cerebral cortices (50–100 mg wet weight) from 2–3 pups sacrificed at PN age 7, 14, 21 and 28 days. The extracts were prepared by homogenizing tissue in a glass homogenizer in 1 ml of buffer (0.01 *M* Na-phosphate pH 7.0, 1 *M* NaCl, 0.05 *M* $MgCl_2$, 1% Triton X-100) at 4°C, incubated at room temperature for 30 min to facilitate solubilization, and centrifuged for 30 min at 10^5 *g* after 30 min. The aliquots of the extracts were saved for total AChE and for protein analysis. Remaining extracts were subjected to sucrose gradient (5–20%) centrifugation to separate the molecular forms of AChE as described [27, 39]. The molecular size of each AChE form was estimated using standards, *Escherichia coli*-galactosidase (16s), catalase (11.4s) and bovine serum albumin (4.5s). The AChE in total extracts and in gradient fractions was determined by Ellman's procedure [2] in the presence of 0.1% Triton X-100 and 10^{-5} *M* ISO-OMPA to inhibit pseudoesterase activity. The protein was determined by the procedure of Lowry modified for the presence of Triton X-100 in the extracts [1].

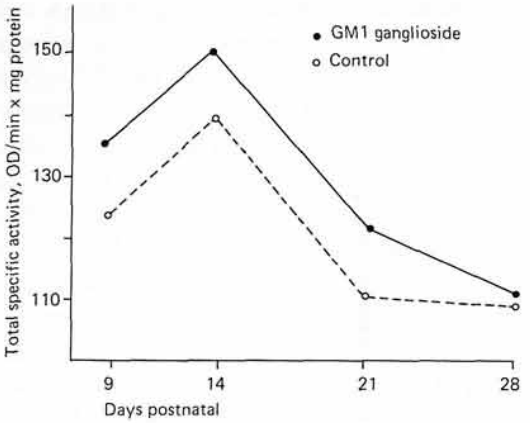

Fig. 2. Total AChE activity as measured in the cortices of animals treated with GM1 ganglioside or injected with saline. GM1-injected rats show consistently higher levels of enzyme activity.

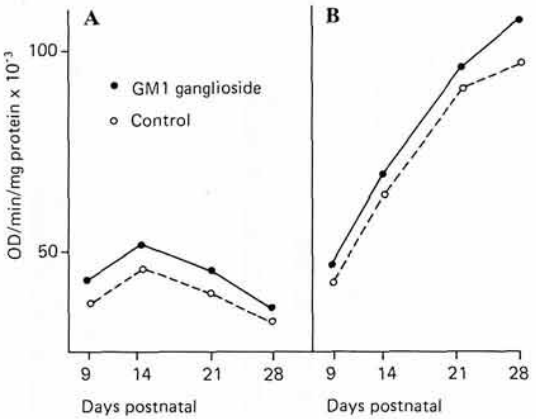

Fig. 3. Levels of 4s **(A)** and 10s **(B)** molecular forms of AChE as measured in the cortices of animals treated with GM1 ganglioside or controls injected with saline. GM1-injected rats show persistently elevated levels of both the 4s and 10s molecular form of the AChE enzyme at all postnatal days monitored.

Results

Behavior

On the first day (PN 11) of training on the avoidance task, rats injected either with total brain ganglioside, GM1 or GD1b ganglioside showed a greater than 50% reduction in escape latencies ($p<0.001$) as compared to controls (saline). Rats injected with either GD1a or GT1b ganglioside were not different from controls (fig. 1).

On the second day of training (PN 12) all experimental groups showed an improvement in their latency scores. Rats injected with either saline, GD1a or GT1b ganglioside improved their performance by reducing their escape latencies by approximately 2s ($p<0.01$) whereas rats injected with either GM1, GD1b or total ganglioside reduced their latencies by approximately 1s ($p<0.05$) (fig. 1).

After skipping 1 day of testing, rats were retested on PN day 14. There was no significant improvement in the performance of the rats injected with either saline, GD1a or GT1b ganglioside. However, rats injected with total ganglioside, GM1 or GD1b ganglioside continued to show improvement in performance of the task ($p<0.05$) (fig. 1).

Rate of body weight gain for all ganglioside-injected rats was identical to that of saline controls. Similarly, day of eye-opening was not observed to be different when comparing ganglioside-injected animals to controls. Preliminary studies where activity levels (Stoelting 6 Station Activity Monitoring System) of total ganglioside or GM1 ganglioside-treated neonates were compared to saline-injected rats showed no differences [12].

Effects of GM1 Ganglioside on AChE

For total AChE activity in the cortices of both GM1 ganglioside-injected rats and saline controls, the specific activity (absorbance at 412 nm/mg protein) increased from PN days 9 to 14 and then decreased (PN days 21 and 28; fig. 2). A similar pattern was seen in measurements of the 4s molecular form of AChE (fig. 3). In contrast, there was a continuous increase in the levels of the 10s molecular form of AChE from PN days 9 to 28 (fig. 3). With one exception, measurements of total AChE activity (fig. 2) and of both molecular forms (4s and 10s) (fig. 3) were consistently higher for rats injected with GM1 ganglioside than for saline controls ($p<0.01$: Friedman Anova). The one exception was total AChE activity on PN day 28 (fig. 2).

Discussion

Behavior

Animals had been trained on the multidirectional escape avoidance task in order to assess their learning performance. Training the animals on PN days 11 and 12 allowed for assessment of the acquisition rate of the behavior. By skipping training on PN day 13 and then testing for performance of the task on PN day 14, data could be further analyzed so as to determine how well the animals had remembered the task (consolidation and retention aspects of learning).

As compared to saline controls, the acquisition of the learned behavior by rats injected with either total brain ganglioside or GM1 or GD1b ganglioside showed an increased ability to acquire the learned task (PN day 11, fig. 1). Rats injected with GD1a or GT1b ganglioside were not different from saline controls. While all groups of animals significantly improved their performance on the second day of training (PN 12), rats treated with total ganglioside, GM1 or GD1b ganglioside continued to perform the task with latency scores which were half those of saline controls. Again the performance of rats injected with either GD1a or GT1b ganglioside was not different from saline-injected rats.

Following 1 day of 'no training', the rats injected with total ganglioside, GM1 or GD1b ganglioside again improved their level of performance (PN 14) as compared to saline controls. In contrast, like the saline controls, rats injected with GD1a or GT1b ganglioside showed no improvement in performance from PN days 12 to 14.

The behavioral data show that those animals injected with brain ganglioside (total, GM1 or GD1b ganglioside) have a greater ability to acquire the learned task (fig. 1, PN 11) as well as an increased ability to retain (retention) the learned behavior. This facilitatory effect of the ganglioside administration on the learning performance of these animals suggests that the maturation of the CNS in these animals may have been in some way either modified or accelerated. No changes have been observed in activity levels, total weight gain or total CNS protein [11] in either total ganglioside or GM1-injected animals, suggesting that there was no enhancement of overall development.

Since gangliosides stimulate neurite outgrowth in vitro and axonal sprouting in vivo, it might be plausible to hypothesize that the exogenous gangliosides are enhancing neuronal maturation, i.e. dendrogenesis and synaptogenesis. Some biochemical evidence supporting this hypothesis is provided by preliminary experiments [20] showing that gan-

glioside injections into rat neonates increase glucose-[^{14}C] incorporation into CNS membranes. Any stimulation of these processes may be reflected in a modulation (in this case facilitation) of CNS functioning as seen in learning performance.

Specificity of the ganglioside's facilitatory effects seems to be indicated since GD1a and GT1b ganglioside were ineffective. This may simply be a matter of the dose administered. If gangliosides were acting as receptors for 'trophic factors', the differences in the effectiveness of the different ganglioside species may reflect the specificity of any given ganglioside species for a particular 'factor'. An analogy for this would be the binding specificity of cholera, tetanus and botulinum toxin for particular ganglioside species [34].

AChE Activity

The overall development patterns (levels) of total AChE activity and the 4s and 10s molecular forms of the enzyme as analyzed in the cortices of the animals tested (GM1-injected and saline control) closely follow levels reported in the literature [27]. The increase in total AChE activity up to PN day 14 parallels reports that the cholinergic system reaches maximal development at about PN 15.

Although there are only small changes in the specific activity levels of the 4s molecular form of AChE from PN days 9 to 28, there is a sustained rise in the specific activity of the 10s form. This observation parallels other research which reports that the ratio of the 10s to the 4s form increases by as much as sevenfold during the first 4 weeks postnatally [27].

The significance of the different molecular forms of AChE has yet to be established. However, since the 10s form increases with maturation and remains high in relation to the 4s form during adulthood, it has been hypothesized that the 10s enzyme reflects 'functional' synapse formation [27]. A heavier enzyme form (17s) associated only with muscle end-plates was not found.

The apparent decrease in the specific activity of AChE (fig. 2) after PN day 14 is partly due to the continuous increase in total brain protein. The evidence also suggests possible endogenous inhibitors for AChE. This evidence is derived from a comparison of the total AChE and the 10s AChE. Although the 10s shows a sustained increase and is the major activity, the total AChE decreases from PN 14 to PN 28. This might be explained by an inhibitor of AChE which is removed during gradient separation of the 4s and 10s forms.

For all three measurements (total AChE, 4s and 10s) the levels found in rats injected with GM1 ganglioside are consistently higher than those of controls. Although the magnitude of the difference is not great, the consistent pattern of treated rats is evident. Although one might conclude that these higher levels of cholinergic enzymes are related to the facilitated learning ability of the neonates, it would be naive to think that the exogenous gangliosides are affecting only the cholinergic system. Analysis of this system was undertaken because of its proposed relation to 'cognitive' function. One anticipates that other transmitter systems would show analogous results.

Both the neurochemical changes and the behavioral results are probably best interpreted as indications of accelerated development rather than simply increases in enzyme activity and behavioral performance. From the data (fig. 2, 3) it is evident that there may be a 3- to 5-day shift (earlier) in the time (PN day) when experimental (GM1) rats reach

maximal enzyme levels as compared to controls. This hypothesis seems tenable since ganglioside effects in vivo on peripheral nerve regeneration (after injury or experimental diabetic neuropathy) have been shown to accelerate axonal sprouting, but not to increase the extent of motor end-plate innervation. If the levels of enzymes were monitored at PN day 60 (i.e., at maturity), one would anticipate them to be indistinguishable from controls and ganglioside-treated animals. In fact, pilot studies show that 60-day-old rats which were treated with GM1 ganglioside according to the same injection protocol showed no improvement in their acquisition and retention performance of a passive avoidance learning behavior.

Our studies show that ganglioside injection (total, GM1 or GD1b ganglioside) of rat neonates results in enhanced neonatal behavioral performance and includes an acceleration in development of the cholinergic system. Though the effects of gangliosides on the cholinergic system may not be specific, the increased levels of this transmitter system may in part account for the enhanced behavioral response. These changes in the levels may represent either an increase in the de novo synthesis or an activation of the enzymes by gangliosides.

The mechanism by which exogenous ganglioside (total, GM1 or GD1b ganglioside) are exerting their effects remain to be established. Though many have suggested gangliosides as possible receptors for 'trophic factors', other hypotheses are also attractive. As proposed by *Purpura and Baker* [26], increased levels of ganglioside might well alter the 'microenvironment' of neuronal membranes in such a way as to stimulate neurite formation (i.e. dendrogenesis and subsequent synaptogenesis).

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Stephen E. Karpiak, PhD,
Division of Neuroscience,
New York State Psychiatric Institute,
722 West 168th Street,
New York, NY 10032 (USA)