

# Effects of Recurrent Hypoglycemia on Brainstem Function in Diabetic BB Rats

## Protective Adaptation During Acute Hypoglycemia

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To determine whether antecedent recurrent hypoglycemia protects the brain from the adverse effects of a standardized hypoglycemic stimulus, we implanted electrodes in the inferior colliculi of diabetic rats to directly record inferior colliculi auditory-evoked potentials (ICEPs). Awake, chronically catheterized BB rats were studied after 2 weeks of insulin therapy designed to produce either chronic hyperglycemia (hyper-DM, glycated hemoglobin  $7.6 \pm 0.4\%$ ) or recurrent hypoglycemia (hypo-DM, glycated hemoglobin  $6.2 \pm 0.7\%$ ), and the results were compared with those observed in nondiabetic rats. When plasma glucose was lowered to and clamped at  $2.8 \text{ mmol/l}$ , the release of catecholamines was suppressed in the hypo-DM rats (epinephrine:  $2.5 \pm 0.4 \text{ nmol/l}$ ) as compared with hyper-DM and the nondiabetic rats ( $9.3 \pm 2.3$  and  $32.7 \pm 6.1 \text{ nmol/l}$ , respectively). ICEP latency was significantly delayed in hyper-DM and nondiabetic rats ( $P < 0.001$ ), but it was unchanged in hypo-DM rats. A more pronounced reduction in plasma glucose ( $2.0 \text{ mmol/l}$ ), however, provoked a greater adrenergic response than that seen at  $2.8 \text{ mmol/l}$  and delayed ICEP latency by 23% in a separate group of hypo-DM animals. These data demonstrate that antecedent recurrent hypoglycemia attenuates the brainstem dysfunction associated with mild to moderate, but not severe, hypoglycemia in diabetic rats. This phenomenon may contribute to the alterations in hypoglycemia counterregulation seen in diabetic patients during intensive insulin therapy. *Diabetes* 48:141-145, 1999

**A**cute hypoglycemia provokes a sequence of events characterized by the secretion of counterregulatory hormones, the appearance of autonomic and neuroglycopenic symptoms, and the development of central nervous system (CNS) dysfunction (1-10). In patients with type 1 diabetes, the status of prior glycemic control may profoundly alter these responses. Hypoglycemia-induced counterregulatory hormone release and/or symptoms tend to occur at a higher plasma glucose

level in chronically hyper-DM patients (11,12), whereas during intensive insulin therapy, the plasma glucose level required to activate these defense mechanisms is lower as compared with nondiabetic controls (13). The latter changes are in large part due to frequent episodes of iatrogenic hypoglycemia (14,15).

It is, however, less clear whether iatrogenic hypoglycemia alters the plasma glucose level at which hypoglycemia-induced brain dysfunction occurs. Intensive insulin therapy associated with iatrogenic hypoglycemia has been reported to protect type 1 diabetic patients from cognitive impairment associated with hypoglycemia in some studies (2,16), but not in others (17). Furthermore, it has been reported that a prior episode of hypoglycemia in nondiabetic volunteers failed to cause an adaptation that protected brain function from a subsequent episode of hypoglycemia (18). These inconsistencies may be related to limitations imposed by indirect measurements of brain function or the inability to maintain prolonged and severe hyperglycemia and/or hypoglycemia in experiments involving human subjects. The spontaneously diabetic BB rat (19), which exhibits much the same alterations in counterregulatory hormone secretion during hypoglycemia (20), offers distinct advantages in this regard.

In previous studies involving direct brainstem recordings from the inferior colliculus of nondiabetic rats, we reported that this region within the auditory pathway is particularly susceptible to the adverse effects of hypoglycemia (21). A similar impairment of brainstem function in the region of the inferior colliculus has also been reported during moderate hypoglycemia in nondiabetic volunteers (6,22). Moreover, in poorly controlled diabetic BB rats, the hypoglycemia-induced impairment of inferior colliculus function occurred at a higher plasma glucose level (23), suggesting greater sensitivity to the adverse effects of hypoglycemia on CNS function.

In this study, we used direct measurements of inferior colliculus auditory-evoked potentials (ICEPs) in the diabetic BB rat to test whether therapy designed to produce recurrent hypoglycemia altered the sensitivity of brainstem function to acute experimental hypoglycemia.

### RESEARCH DESIGN AND METHODS

**Animal models.** Spontaneously diabetic male BB/Wor rats (~75 days duration) and age-matched nondiabetic diabetes-resistant BB control rats were obtained from the University of Massachusetts breeding facility. All animals were housed in the Yale Animal Care Facility, kept on a 12-h/12-h day/night cycle, and fed a standard ad libitum rat food diet consisting of 51% carbohydrate, 5% fat, and 22% protein, with the remaining weight accounted for by moisture and nonmetabolizable solids, such as ash (Agway Prolab 3000).

The diabetic animals were divided into two treatment groups. Group 1 diabetic rats (hyper-DM,  $n = 6$ ) were treated once daily at 1600-1700 with subcu-

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BAEP, brainstem auditory-evoked potential; CNS, central nervous system; hyper-DM, hyperglycemic diabetic; hypo-DM, hypoglycemic diabetic; ICEP, inferior colliculus auditory-evoked potential.

taneous injections of ~8 U/kg protamine zinc insulin (Lilly, Indianapolis, IN), which was designed to maintain body weight ( $294 \pm 14$  g) but inadequate to prevent hyperglycemia. Random plasma glucose levels were measured via tail vein two to three times each week to ensure that the insulin treatment produced chronic hyperglycemia and avoided hypoglycemia for 2 weeks before study. Group 2 diabetic rats ( $n = 13$ ;  $375 \pm 8$  g) were treated with twice-daily injections of protamine zinc insulin in amounts ( $\sim 12 \text{ U} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ ) required to produce 2 weeks of recurrent hypoglycemia (hypo-DM). The evening dose of insulin was adjusted to produce moderate hypoglycemia by 0900, and the morning injection maintained decreased levels until at least 1600. In addition, a group of six nondiabetic control animals ( $368 \pm 10$  g) were studied without prior insulin treatment. Data from the control group have been reported previously (23), but the control data were generated at approximately the same time as the current data in the diabetic rats.

One week before study (at which time the diabetic rats had been on their insulin regimen for at least 7 days), the animals were anesthetized using ketamine ( $60 \text{ mg/kg i.m.}$ ) and pentobarbital ( $21 \text{ mg/kg i.p.}$ ). Catheters were positioned in an artery and a central vein for later use, and microelectrodes were stereotaxically placed bilaterally in the inferior colliculus region of the brain and on the skull, as previously described (21). Only those animals whose weight had stabilized after surgery and in whom glucose levels during the postoperative week were in the appropriate range were subsequently used in the infusion protocols described below. These studies were approved by the animal care and use committee of Yale University School of Medicine.

**Experimental design.** Insulin doses in the recurrently hypoglycemic group were reduced the day before study to allow plasma glucose in these animals to return to hyperglycemic levels overnight. This permitted both groups of diabetic rats to be matched with respect to glucose levels at the initiation of the hypoglycemic challenge the following day. On the morning of each experiment, catheters were flushed and microelectrodes were connected to a brain wave analyzer (Compact 4; Nicolet Biomedical Systems, Madison, WI), as described previously (21). Blood was collected to measure glycated hemoglobin; food and water were removed for the duration of the study. Thereafter, animals were allowed free movement in their cages for 1 h before study so they could relax from the stress of handling.

Before initiating the hypoglycemic insulin clamp protocols, at least two sets of ICEPs were measured under basal conditions over a 20-min interval. In addition, traditional brainstem auditory-evoked potentials (BAEPs) were measured in the basal state to assess overall auditory pathway function and exclude adverse effects caused by the surgical placement of the electrodes. Conventional BAEPs, recorded using electrodes attached to the skull, measure the response of the lower brainstem (which includes the inferior colliculus) to an auditory stimulus. The placement of depth electrodes (ICEP) allowed us to focus with greater sensitivity on a well-defined region of the auditory brainstem that appears to be glucose-sensitive. It is important to bear in mind that the inferior colliculus represents a segment of the primary CNS response to auditory stimuli before any cognitive components of the sensory cortex become involved (such as during a P300 measurement).

Blood samples were also collected for measurement of plasma catecholamines and glucose. Thereafter, a primed ( $336 \text{ pmol/kg}$  over 5 min) plus continuous ( $84 \text{ pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) infusion of regular porcine insulin (Eli Lilly) was begun and continued for 210 min. Plasma glucose was allowed to decline to 2.8 mmol/l and was maintained there by a variable infusion of 20% dextrose based on 5- to 10-min plasma glucose determinations in the following groups: hyper-DM rats ( $n = 6$ ), hypo-DM rats ( $n = 7$ ), and nondiabetic rats ( $n = 6$ ). In addition, a separate group of hypo-DM rats received an identical hypoglycemic clamp study, except that plasma glucose was lowered further to 2 mmol/l ( $n = 6$ ). During each experiment, plasma glucose, catecholamines, and ICEPs were measured at 90, 150, and 210 min after the start of the insulin infusion. Blood loss due to sampling ( $<4 \text{ ml}$ ) was replaced using donor blood from a nondiabetic control rat.

**Analytical measurements and calculations.** Results of brain function analyses are displayed as latency of response for each peak (milliseconds). Postinfusion histological examination was used to verify the correct placement of inferior colliculus electrodes.

Plasma glucose was measured using a Beckman glucose analyzer (Fullerton, CA). Plasma epinephrine and norepinephrine were assayed radioenzymatically (Amersham, Arlington Heights, IL). Glycated hemoglobin was determined using an affinity column procedure (Isolab, Akron, OH). All data are expressed as means  $\pm$  SE and analyzed using analysis of variance followed by post hoc Bonferroni corrected *t* test to localize repeated measures differences (SYSTAT; SPSS, Chicago).

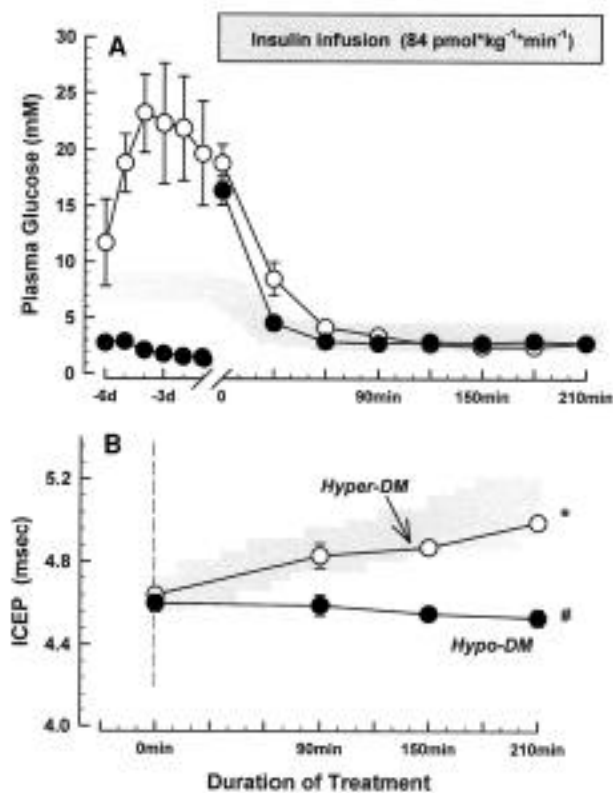
## RESULTS

**Glucose levels in three groups.** Plasma glucose levels in each of the experimental groups after surgical catheterization and microelectrode placement are shown in Fig. 1. In hypo-DM rats, plasma glucose concentrations in the 7-day period

before study consistently fell to  $<2.8 \text{ mmol/l}$  at least once during the day between 0900 and 1700. Glucose levels averaged  $2.2 \pm 0.2 \text{ mmol/l}$  during this 6- to 8-h interval. Moreover, glycated hemoglobin levels in the hypo-DM rats ( $6.2 \pm 0.7\%$ ) were nearly identical to nondiabetic control levels ( $5.9 \pm 0.6\%$ ). As shown in Fig. 1, when insulin was withdrawn on the day before study, there was recovery from hypoglycemia on the morning of the experiment so that plasma glucose levels in the hypo-DM group ( $13.8 \pm 1.4 \text{ mmol/l}$ ) were not significantly different from those of the hyper-DM group. Both random morning glucose levels ( $19.2 \pm 3.1 \text{ mmol/l}$ ) and glycated hemoglobin levels ( $7.6 \pm 0.4\%$ ) were higher in the hyper-DM group compared with the nondiabetic control group.

During the insulin infusion, glucose concentrations fell to the target level ( $2.8 \text{ mmol/l}$ ) within 30–45 min in the recurrently hypoglycemic group and in the untreated nondiabetic rats (depicted as a shaded region). The hyper-DM animals required a slightly longer time period to reach the desired plateau ( $\sim 60$  min). Thereafter, all groups were maintained at a comparable level of hypoglycemia for at least 2 h ( $2.7 \pm 0.1$ ,  $2.8 \pm 0.1$ , and  $2.9 \pm 0.1 \text{ mmol/l}$  in hyper-DM, hypo-DM, and nondiabetic rats, respectively).

**Counterregulation during moderate hypoglycemia.** Figure 2 shows basal and peak response epinephrine and norepinephrine concentrations during moderate hypoglycemia



**FIG. 1.** Plasma glucose and ICEP in chronically hyperglycemic and recurrently hypoglycemic rats. **A:** Plasma glucose levels during the final 7 days of treatment (–6 to 0 days) and during a 2.8 mmol/l hypoglycemic clamp (0–210 min) in two groups of diabetic rats and nondiabetic control rats (shown as the shaded range). **B:** Latency of ICEP in three groups of rats during the clamp procedure. Data are expressed as means  $\pm$  SE; \* $P < 0.001$  vs. basal, # $P < 0.001$  vs. hyper-DM rats and nondiabetic rats. Data from nondiabetic control rats have been presented previously (23).

(2.8 mmol/l) in all groups. Plasma epinephrine was comparable in the hyper-DM and nondiabetic groups in the basal state ( $0.69 \pm 0.22$  and  $0.96 \pm 0.21$  nmol/l, respectively), whereas the hypo-DM group exhibited reduced epinephrine levels ( $0.26 \pm 0.05$ ,  $P < 0.05$  vs. nondiabetic). During moderate hypoglycemia, plasma epinephrine rose in the hyper-DM group to  $9.29 \pm 2.34$  nmol/l during the last hour of hypoglycemia. This response was nevertheless decreased by 72% as compared with that observed in the nondiabetic control group ( $32.66 \pm 6.09$  nmol/l,  $P < 0.001$ ). More pronounced suppression of the plasma epinephrine response was observed in diabetic rats that had been exposed to recurrent hypoglycemia ( $2.49 \pm 0.44$ ,  $P < 0.05$  vs. nondiabetic and hyper-DM rats).

Norepinephrine was slightly, but not significantly, elevated in diabetic rats on the morning of study ( $3.08 \pm 0.31$  in hyper-DM rats and  $2.61 \pm 0.64$  in hypo-DM rats) as compared with nondiabetic control rats ( $2.18 \pm 0.19$ , NS). Norepinephrine levels rose modestly during hypoglycemia in hyper-DM rats ( $4.65 \pm 0.51$ ,  $P < 0.05$  vs. basal) but not in hypo-DM rats ( $3.27 \pm 0.26$  nmol/l, NS). In contrast, the nondiabetic rats showed a two- to fourfold increase in plasma norepinephrine above baseline during hypoglycemia (to  $6.75 \pm 1.09$ ,  $P < 0.02$  vs. basal).

**Brain function during moderate hypoglycemia.** Despite marked differences in treatment glucose levels, baseline ICEP latency was similar in all groups ( $\sim 4.6$  ms); no baseline alterations attributable to treatment or diabetes were observed (Fig. 1). Moreover, all aspects of conventional BAEPs were

comparable to those in normal rats before surgery, indicating that the surgical model in the present study was not associated with alterations in brainstem function. Furthermore, BAEPs were similar among the three groups before the insulin infusion protocols (data not shown), suggesting that diabetes per se had not altered baseline auditory brainstem function.

In the hypoglycemic phase of the study, chronically hyper-DM rats showed a marked impairment in ICEP latency after 90 min (from  $4.64 \pm 0.02$  to  $4.83 \pm 0.06$  ms,  $P < 0.02$  vs. basal). This response deteriorated further by 210 min (to  $4.99$  m,  $P < 0.001$  vs. basal). The pattern and magnitude of these changes in hyper-DM rats were equivalent to that seen in nondiabetic control animals (depicted as a shaded region in Fig. 1). In sharp contrast, hypo-DM rats did not exhibit any impairment of ICEP latency during moderate hypoglycemia ( $4.53 \pm 0.04$  at 210 min vs.  $4.60 \pm 0.04$  ms at baseline).

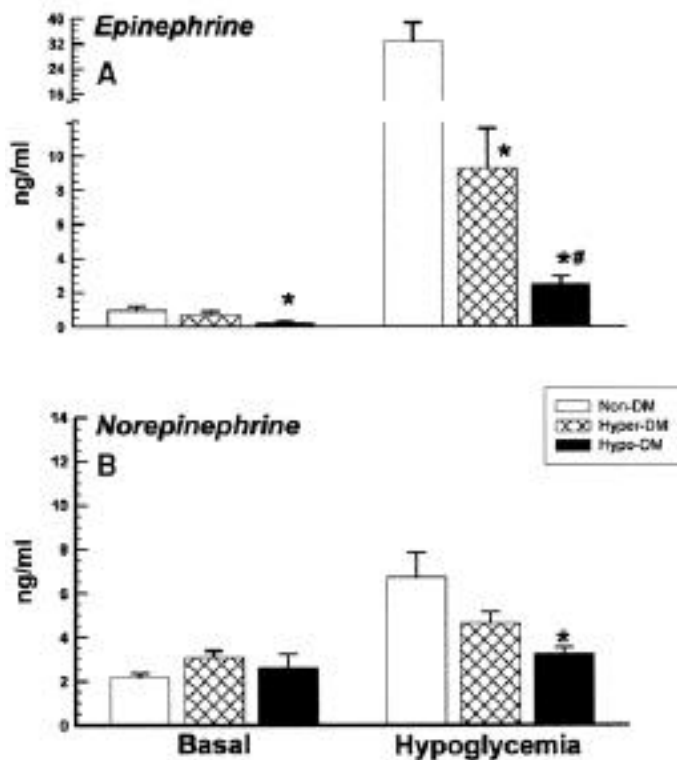
**Counterregulation and brain function during more severe hypoglycemia.** During the experiments in hypo-DM rats, in which plasma glucose was lowered to  $\sim 2.0$  mmol/l, there was a sharp 6.5-fold increase in plasma epinephrine (to  $15.58 \pm 2.44$  nmol/l). Nevertheless, this response was only 50% of that observed in healthy nondiabetic control rats during less severe hypoglycemia ( $P < 0.05$  vs. nondiabetic at 2.8 mmol/l glucose). The norepinephrine response to this more severe hypoglycemic stimulus (to  $8.87 \pm 1.75$  nmol/l) was comparable to that observed in nondiabetic control rats studied at 2.8 mmol/l.

This more severe hypoglycemic stimulus produced a significant impairment in inferior colliculus function in the recurrently hypo-DM rats (Fig. 3). ICEP latency increased significantly by  $\sim 6\%$  within 90 min (to  $4.94 \pm 0.09$  ms) and continued to deteriorate throughout the study ( $5.77 \pm 0.14$ ).

## DISCUSSION

Hypoglycemia remains the major factor limiting the application of intensive insulin therapy designed to prevent complications in patients with type 1 diabetes (24,25). This phenomenon is explained, at least in part, by a therapy-induced lowering of the glucose level at which adrenergic responses and symptoms are triggered (13,26), presumably due to more frequent iatrogenic hypoglycemia (15). It is noteworthy that similar suppression of adrenergic responses was also seen in recurrently hypoglycemic animals in the current study. A key unresolved clinical issue is whether hormone release after intensive insulin therapy is accompanied by a similar downward shift in the glucose level at which brain function becomes impaired. Previous studies examining this issue in diabetic patients have yielded conflicting data (2,15,16).

To overcome some of the technical limitations of measuring subtle changes in brain function in humans during mild to moderate hypoglycemia, we monitored inferior colliculus function directly in awake diabetic BB animals. The function of this region of the brainstem has previously been shown to be adversely affected by relatively small decrements in circulating glucose (21,23). In previous experiments combining the hypoglycemic clamp with measurements of auditory evoked potentials directly recorded from the inferior colliculus, we showed that when plasma glucose was lowered to  $\sim 3.5$  mmol/l, the evoked potential was delayed in chronically hyper-DM BB rats, but not in nondiabetic control rats (23). In the control rats, a greater reduction of plasma glucose (i.e., 2.8 mmol/l) was required to impair inferior colliculus function.



**FIG. 2.** Plasma counterregulatory hormones before and during moderate hypoglycemia. Epinephrine (A) and norepinephrine (B) levels were measured basally and at the peak response during a 2.8 mmol/l hypoglycemic clamp in diabetic and nondiabetic rats. \*  $P < 0.02$  vs. nondiabetic rats, #  $P < 0.005$  vs. hyper-DM rats.

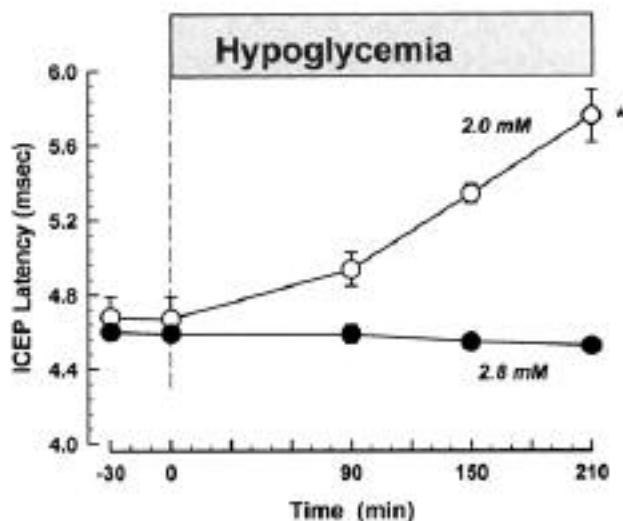


FIG. 3. Effects of more severe hypoglycemia on ICEP latency in recurrently hypo-DM rats. \* $P < 0.001$  vs. 2.8 mmol/l group.

These findings suggest that this brainstem function is more susceptible to mild hypoglycemia in poorly controlled hyper-DM rats.

In the current study, we examined the converse situation, namely, the effect of 2 weeks of recurrent iatrogenic hypoglycemia on the glycemic level at which auditory evoked potentials become impaired. Our data demonstrate that the deliberate induction of recurrent hypoglycemia did more than merely correct an adaptation observed in hyper-DM animals, it maintained normal brainstem function during hypoglycemia to a greater extent than was seen in control animals. It is noteworthy that while the brainstem function was relatively resistant to the adverse effects of mild to moderate hypoglycemia, with more severe hypoglycemia, marked functional deterioration occurred. These findings suggest that the adaptive response of the brain in these diabetic rats is limited, and that once plasma glucose falls below values that can be compensated for, neuroglycopenia rapidly ensues. Similar results have been reported in type 1 diabetic patients receiving intensive insulin therapy in whom cortical evoked potentials were used to assess brain function during hypoglycemia (16,22). Moreover, the data are consistent with clinical evidence of a substantially higher rate of severe hypoglycemia in intensively treated patients.

Although the mechanism responsible for the relative resistance to neuroglycopenia observed in our recurrently hypo-DM rats is uncertain, recent data suggest that this phenomenon may be at least in part a consequence of more efficient blood-brain barrier glucose transport (27,28). In keeping with this interpretation, recurrent hypoglycemia in normal and diabetic patients increases brain glucose utilization (29,30) and can lead to overexpression of blood-brain barrier glucose transporter protein (31). Moreover, several days of sustained hypoglycemia in nondiabetic volunteers or intensive insulin therapy in diabetic patients has been reported to prevent the decrement in brain glucose uptake normally seen during acute hypoglycemia (29,30).

In summary, recurrent hypoglycemia in diabetic BB rats protects the function of the inferior colliculus region of the

brainstem during mild to moderate but not during severe hypoglycemia. To the extent that this brainstem region reflects other brain functions, the suppression of hypoglycemic counterregulatory responses induced by iatrogenic hypoglycemia may reflect in part an adaptive response to the development of CNS resistance to neuroglycopenia. Unfortunately, the capacity of this brain adaptation is limited and thus may ultimately become maladaptive in the clinical setting of severe hypoglycemia.

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