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Nrf2-Mediated Neuroprotection Against Recurrent Hypoglycemia Is Insufficient to Prevent Cognitive Impairment in a Rodent Model of Type 1 Diabetes

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It remains uncertain whether recurrent nonsevere hypoglycemia (Hypo) results in long-term cognitive impairment in type 1 diabetes (T1D). This study tested the hypothesis that specifically in the T1D state, Hypo leads to cognitive impairment via a pathological response to oxidative stress. Wild-type (Control) and nuclear factor–erythroid 2 p45–related factor 2 (Nrf2) null mice were studied. Eight groups of mice (Control and *Nrf2*$^{-/-}$ ± T1D and ± Hypo) were subject to recurrent, twice-weekly, insulin or saline injections over 4 weeks, after which cognitive function was assessed and brain tissue analyzed. Recurrent moderate hypoglycemia in T1D, but not Control, mice significantly impaired cognitive performance, and this was associated with hippocampal oxidative damage and inflammation despite an enhanced expression of Nrf2 and its target genes *Hmox1* and *Nqo1*. In *Nrf2*$^{-/-}$ mice, both T1D and Hypo independently resulted in impaired cognitive performance, and this was associated with oxidative cell damage and marked inflammation. Together, these data suggest that Hypo induces an Nrf2-dependent antioxidant response in the hippocampus, which counteracts oxidative damage. However, in T1D, this neuroprotective mechanism is insufficient to prevent neuronal oxidative damage, resulting in chronic deficits in working and long-term memory.

Hypoglycemia is a common adverse side effect of insulin therapy in type 1 diabetes (T1D), largely due to hyperinsulinemia, a diminished counterregulatory response, and impaired awareness of hypoglycemia (1). The brain is especially vulnerable to hypoglycemia due to its high metabolic demand and minimal fuel stores. As such, there is potential for recurrent hypoglycemia to produce long-term neuronal damage. This is a source of major concern and fear for individuals with T1D (2), especially as studies in animals and humans have yielded inconsistent findings.

Profound hypoglycemia, sufficient to cause coma, results in brain damage in humans (3,4), but epidemiological studies yield conflicting data on the impact of reversible severe and nonsevere hypoglycemia on long-term cognitive function (5). For instance, 18 years' follow-up of T1D individuals in the Diabetes Control and Complications Trial (DCCT) (6) found no evidence of an association between severe hypoglycemia and cognitive decline. In contrast, prospective studies in prepubertal children with T1D have reported that severe hypoglycemia may result in long-term neurological damage and psychomotor retardation (7–11). A limitation of these studies is that the cognitive decline resulting from recurrent hypoglycemia may take place over many decades, and accurate documentation of the frequency of hypoglycemia over such timescales is extremely difficult.

Studies in animal models offer the opportunity to address many of these questions over shorter time frames as well as to examine underlying mechanisms. This literature though is equally conflicting. Again, profound hypoglycemia (sufficient to induce an isoelectric electroencephalogram

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and/or multiple seizures) in animal models causes brain damage particularly in hippocampus and frontal cortex (12–15). However, significant neuronal damage under such conditions would be anticipated, and the relevance therefore to T1D in which hypoglycemia of such severity is rare is not clear. In contrast, nonsevere hypoglycemia does not appear to induce neuronal cell death (16), and long-term recurrent nonsevere hypoglycemia has even been shown to protect against age-related cognitive decline (17). In addition, recurrent nonsevere hypoglycemia in rodents potentially preconditions the brain, protecting it to some extent from neurological damage resulting from subsequent very severe hypoglycemia (18).

An additional limitation of studies in animals is that many were primarily conducted using nondiabetic models. This is important, because in T1D, intermittent exposure to hypoglycemia will always occur in the context of chronic hyperglycemia of varying degrees. Chronic hyperglycemia (19), severe hypoglycemia (20,21), and glucose recovery from hypoglycemia (13,22) have each independently been shown to stimulate reactive oxygen species (ROS) production. In addition, chronic hyperglycemia may impair antioxidant defense mechanisms (23–25). Therefore, the ability of neurons to respond to nonsevere hypoglycemia may be uniquely impaired in T1D increasing vulnerability of the brain particularly to oxidative stress, but this question has to date not been addressed.

To test this hypothesis, we studied insulin-treated T1D and nondiabetic rodent models that were exposed to intermittent episodes of nonsevere hypoglycemia over 4 weeks and examined the impact of these interventions on cognitive function and markers of oxidative stress and inflammation. Having demonstrated that recurrent hypoglycemia in T1D but not nondiabetic rodents induced defects in cognitive function that were associated with hippocampal inflammation and oxidative damage, we subsequently sought to clarify the role of the oxidative stress response by studying mice lacking the transcription factor nuclear factor–erythroid 2 p45–related factor 2 (Nrf2) because it plays a critical role in regulating basal cellular antioxidant defenses as well as orchestrating responses to oxidative stress (for review, see Ref. 26). In this study, we report that *Nrf2*$^{-/-}$ mice are very vulnerable to both T1D and recurrent hypoglycemia and that loss of Nrf2 resulted in strongly enhanced hippocampal inflammatory and oxidative damage responses to these two metabolic stimuli.

RESEARCH DESIGN AND METHODS

Experimental Animals

Sixty-four adult male C57BL/6J mice (20–25 g; Charles River Laboratories, U.K.) and 30 male *Nrf2*$^{-/-}$ (20–25 g) mice were used. Generation and genotyping of *Nrf2*$^{-/-}$ mice (provided by Ken Itoh and Masayuki Yamamoto, Centre for Tsukuba Advanced Research Alliance and Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Japan) have been described previously (27). Animals were fed ad libitum, on a 12:12-h light/dark schedule. All animal procedures were approved by the University of Dundee Ethical Review Process and performed in accordance with U.K. Home Office regulations (under the auspices of Project License PIL60/4120).

Experimental Groups

Groups of animals were randomly assigned to receive streptozotocin (STZ) (125 mg/kg i.p.) to induce T1D or control (citrate acid buffer i.p.). Tail vein blood glucose (Accu-Read; Accu-Read, Guangdong, China) was measured 3 and 7 days post-STZ and a reading ≥16.0 mmol/L (288 mg/dL) regarded as diabetic. Animals failing to reach this were given a second injection of STZ and retested as above. Mice were subsequently subdivided into recurrent hypoglycemia (Hypo) or control, giving the following four test groups: *1*) Control, *2*) Control + Hypo, *3*) T1D, and *4*) T1D + Hypo (n = 8/group) (Supplementary Fig. 1*A*). Similarly, for studies of *Nrf2*-null mice, animals were randomly allocated to the following four test groups: *1*) *Nrf2*$^{-/-}$, *2*) *Nrf2*$^{-/-}$ + Hypo, *3*) T1D *Nrf2*$^{-/-}$, and *4*) T1D *Nrf2*$^{-/-}$ + Hypo (n = 7 to 8/group).

Surgery

Animals were anesthetized by isoflurane and Linbit insulin implants inserted subcutaneously. Insulin replacement was used in an attempt to replicate more closely human insulin-treated T1D and to ensure the animals remained healthy and showed positive weight trajectory over the 12 weeks of the experiment (Supplementary Fig. 1*B* and *C*). However, for the purposes of this study in which the interaction between chronic hyperglycemia and Hypo was being explored, insulin implants at half of the recommended dose (~0.05 U/kg/day) were used (Fig. 1*A* and *C*). Control animals were also anesthetized and subjected to sham surgery.

Recurrent Hypoglycemia

Mice were subjected to eight episodes of hypoglycemia (two per week for 4 weeks) (Supplementary Fig. 1*A*). Following a 4-h fast, basal glucose was measured from the tail vein, and insulin was injected (0.75 mU/g *Nrf2*$^{-/-}$, 1 mU/g Control and wild-type [WT] animals and 4 mU/g T1D and T1D *Nrf2*$^{-/-}$ i.p.) to induce moderate hypoglycemia (2.5 –3 mmol/L) (Fig. 1*B* and *D*). Hypoglycemia was maintained for 2 h and animals allowed to return to euglycemic levels with food. Animals were monitored continuously during the hypoglycemic period. After 2 weeks, insulin doses were reduced to 0.5 mU/g *Nrf2*$^{-/-}$, 0.75 mU/g Control animals, and 2.5 –3 mU/g for T1D and T1D *Nrf2*$^{-/-}$ animals, respectively. Any animal showing signs of physical impairment received glucose (1 mg/kg i.p.). Two animals in the Control group, one animal in the T1D group, and one animal from the *Nrf2*$^{-/-}$ group required recovery on one occasion. Control animals were fasted and given saline injections. No animals suffered from seizures.

Behavioral Procedures

Cognition was assessed by novel object recognition (NOR) and spontaneous alternation tests (28,29), at least 3 days after the last hypoglycemic episode (Supplementary Fig. 1*A*). Beckman activity box and open field maze tasks were

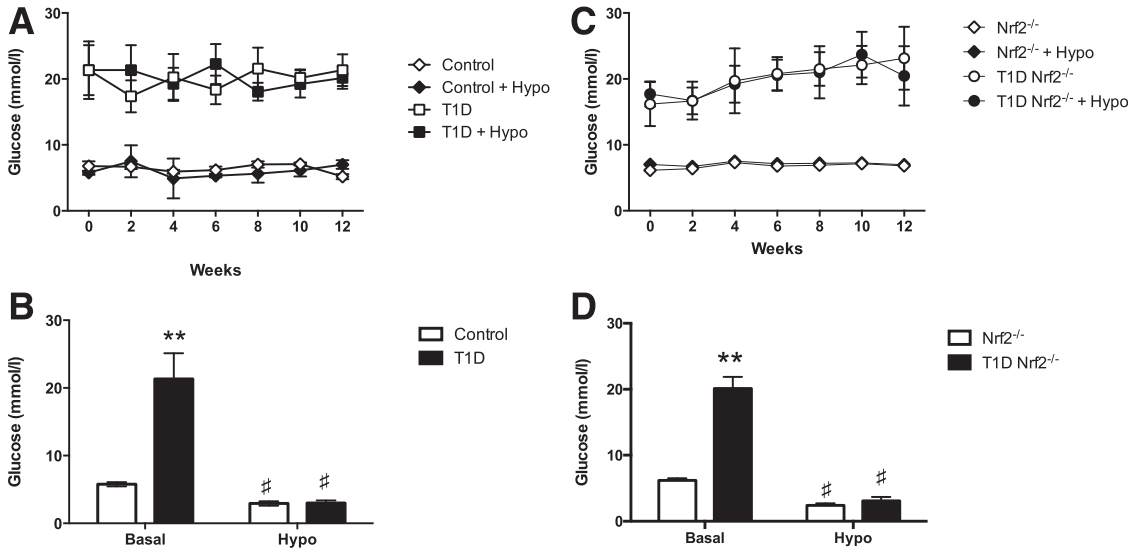

Figure 1—Physiological profile of diabetic and nondiabetic WT and *Nrf2*$^{-/-}$ mice exposed to recurrent hypoglycemia. *A* and *C*: Levels of hypoglycemia were comparable between experimental groups and unaltered by either genotype or recurrent hypoglycemia. *B* and *D*: Example of typical insulin-induced hypoglycemic event in WT and *Nrf2*$^{-/-}$ animals with a comparable level of hypoglycemia achieved despite *Nrf2*$^{-/-}$ animals receiving significantly lower insulin doses (starting dose 0.75 mU/g WT Control vs. 0.5 mU/g *Nrf2*$^{-/-}$ animals). Control, Control + Hypo, T1D, and T1D + Hypo, all n = 8/group. *Nrf2*$^{-/-}$, *Nrf2*$^{-/-}$ + Hypo, T1D *Nrf2*$^{-/-}$, and T1D *Nrf2*$^{-/-}$ + Hypo, all n = 7 to 8/group. Results represent mean values ± SEM. Data were analyzed by two-way ANOVA with T1D and Hypo as between-subject factors followed by Tukey post hoc test. $^{**}P < 0.01$ Control or *Nrf2*$^{-/-}$ vs. T1D or T1D *Nrf2*$^{-/-}$, $\#P < 0.05$.

also made to exclude any confounding effects of altered activity or anxiety.

NOR

A simple hippocampal-mediated task based on the innate tendency of rodents to seek novelty was used (28). The primary outcome measure was the discrimination index (D3), calculated as the total time spent exploring the familiar objects/total time spent exploring the novel object, for short-term (10-min) and long-term (24-h) memory.

Spontaneous Alternation

This was used to test spatial working memory as described previously (29). Memory was assessed as a percent 4/5 alternation with an alternation counted when all four arms are visited within a span of five arm entries (29).

Activity Box

Locomotor activity was assessed using a Beckman activity box. Animals, habituated to the box for 4 days, were placed into the box and allowed to explore freely for 15 min. Mobile, active, and static counts were recorded.

Open Field Maze

Activity was recorded on film and later analyzed by two independent scorers for total time spent within the inner and outer zones of the maze. Activity is presented as percent total time spent in each zone.

Biochemical Analyses

On completion of behavioral testing, animals were killed humanely, brain tissue dissected, and flash frozen in liquid nitrogen for subsequent biochemical analyses.

Lipid Peroxidation

The concentration of malondialdehyde was determined in hippocampus using the thiobarbituric acid–reactive substances assay as described in Mihara and Uchiyama (30) and adapted for a 96-well plate format. The amount of malondialdehyde in the samples was determined spectrophotometrically at 532 nm and concentration determined from a standard curve. All samples were assayed in duplicate.

Protein Carbonylation

Levels of carbonylated protein within the hippocampus were measured by ELISA (Cayman Chemical). Protein carbonyl concentration was calculated using the following equation: protein carbonyl (nmol/mL) = ([CA]/[0.011 μmol/L^{-1}]) (500 μL/200 μL), where CA is equal to the corrected absorbance (average absorbance of controls − average absorbance of samples).

Proinflammatory Protein Array

The total protein levels of a panel of inflammatory cytokines (interferon-γ, interleukin [IL]-10, IL-12p70, IL-1β IL-2, IL-4, IL-5, IL-6, keratinocyte chemoattractant/human growth-related oncogene, and tumor necrosis factor-α [TNF-α]) were measured using the V-Plex Proinflammatory Panel (mouse) kit (Meso Scale Discovery). Hippocampal homogenates (50 μg) were assayed in duplicate and values presented relative to their appropriate Control group (Control or *Nrf2*$^{-/-}$, respectively).

RNA Extraction and PCR

Total RNA was extracted from hippocampal tissue using TRIzol reagent (Invitrogen). Reverse transcription was performed with 1 ng RNA using SuperScript III First

Strand Synthesis system for RT (Invitrogen). Real-time PCR was performed using TaqMan gene expression assays for the following genes: Nrf2 and Nrf2 target genes [*Hmox1*, *Nqo1*, *Gsta1*, *Txn1*, *Txrnd1*, and *Srxn1* (31)], the inflammatory genes *IL-1β*, *IL-6*, *Tnfα*, and *Nos2*, and housekeeping genes (Applied Biosystems) (see Supplementary Table 1 for full details of primer/probe sequences). All samples were performed in triplicate and normalized to actin. Values are expressed as a fold-change relative to Control.

Statistical Analysis

Data were analyzed using SPSS version 18 (SPSS, Chicago, IL). Multivariate ANOVA was used to compare groups with treatment (TID) or genotype ($Nrf2^{-/-}$) and Hypo as between subject variables. Post hoc analysis was performed using the Tukey multiple-comparisons test. Data are expressed as mean values ± SEM. Statistical significance was set at $P < 0.05$.

RESULTS

Recurrent Hypoglycemia in Wild-type and $Nrf2^{-/-}$ Mice With or Without T1D

As expected, blood glucose levels were higher in T1D compared with WT animals over the 12-week study (Fig. 1*A*) (T1D × week $F[3.74,93.48] = 35.89$; $P < 0.05$). However, the level of hypoglycemia achieved was comparable between T1D and Control groups (Fig. 1*B*) (average glucose, Control = 2.9 ± 0.3 vs. TID = 3.0 ± 0.4 mmol/L; $t = 0.088$; $P = 0.97$) and did not change over the duration of the experiment (T1D, $F[1,26] = 0.621$; $P = 0.45$).

Similarly, T1D $Nrf2^{-/-}$ mice had significantly higher fasting glucose levels when compared with nondiabetic $Nrf2^{-/-}$ animals for the duration of the study (Fig. 1*C*) (T1D × week, $F[3.66,36.66] = 21.53$; $P < 0.01$) but the degree of hypoglycemia achieved between T1D $Nrf2^{-/-}$ and $Nrf2^{-/-}$ animals was comparable (Fig. 1*D*) (average glucose, $Nrf2^{-/-}$ = 2.4 ± 0.3 vs. TID $Nrf2^{-/-}$ = 3.1 ± 0.6 mmol/L; $t = 0.062$, $P = 0.95$).

Recurrent Hypoglycemia Impairs Cognitive Function in T1D Wild-type and $Nrf2^{-/-}$ Mice

The most significant cognitive defect found was in long-term memory as assessed by the NOR task in T1D + Hypo mice (Fig. 2*A*) (T1D × Hypo, $F[1,30] = 5.936$; $P < 0.05$). A less marked cognitive defect at this time point was also found in T1D mice (Fig. 2*A*) (T1D, $F[1,30] = 52.40$; $P < 0.05$), but not in Control or Control + Hypo animals. These differences were not due to a reduction in time spent exploring the objects (effect of T1D and Hypo, both P = not significant). Performance on the NOR task after a 10-min interval was intact in all groups (main effects T1D and Hypo on D3 indices and Exploration time; all P = not significant), indicating that both the ability to learn the task and short-term memory for objects were intact.

On the spontaneous alternation task, T1D + Hypo animals were also most affected, demonstrating a reduction in percentage alternations ([total alternations/total entries − 4] * 100) (Fig. 2*B*) (T1D × Hypo, $F[1,15] = 5.71$; $P < 0.01$) and fewer total alternations (T1D × Hypo, $F[1,15] = 7.734$; $P < 0.01$; average total alternations; Control 46.29 ± 3.66, Control + Hypo 48.36 ± 3.63, T1D 47.10 ± 4.67, and T1D + Hypo 41.67 ± 3.80). In contrast, Control animals receiving Hypo showed a trend toward enhanced percentage of alternation (Fig. 2*B*) ($F[1,15] = 3.04$; $P = 0.10$). There was no effect of T1D alone on the total number of entries ($F[1,30] = 2.92$; P = not significant).

As with wild types, all $Nrf2^{-/-}$ animals performed the short-term NOR task above chance levels (D3 index of ≥0.2; $F < 1$; P = not significant), and as before, there were no differences between groups in discrimination indices and time spent exploring the objects (D3 indices and Exploration time; all $F < 1$; P = not significant). However, after 24 h, only Control $Nrf2^{-/-}$ animals were able to perform the task with both Hypo, T1D, and Hypo + T1D markedly impairing long-term memory (Fig. 2*C*) (Hypo, $F[1,25] = 12.09$, $P < 0.01$; T1D, $F[1,25] = 6.95$, $P < 0.05$; and T1D × Hypo, $F[1,25] = 6.24$, $P < 0.05$). These differences were not due to a reduction in time spent exploring the objects (Exploration time; all $F < 1$; P = not significant).

In both nondiabetic and T1D $Nrf2^{-/-}$ animals, Hypo resulted in impaired working memory ([total alternations/total entries − 4] * 100) (Fig. 2*D*) (Hypo $F[1,25] = 18.18$; $P < 0.01$; $F < 1$ for the effect of T1D and T1D × Hypo). This impairment was accompanied by a significant reduction in the total number of alternations (Hypo $F[1,25] = 9.22$, $P < 0.01$; T1D, $F[1,25] = 17.28$, $P < 0.01$; $F < 1$, P = not significant for effect of T1D × Hypo; average total alternations: $Nrf2^{-/-}$ 28.35 ± 2.78, $Nrf2^{-/-}$ + Hypo 21.00 ± 2.80, T1D $Nrf2^{-/-}$ 24.86 ± 4.11, and T1D $Nrf2^{-/-}$ + Hypo 10.14 ± 1.83). As with wild-type mice, there was no difference in total number of entries (T1D, $F[1,25] = 4.12$; $P = 0.776$; $F < 1$, P = not significant for effect of Hypo and T1D × Hypo).

Assessments of locomotor activity and anxiety on the open field maze (data not shown) did not differ between any groups (active, mobile, or static counts [all $F < 1$]; time spent in central inner zone or the outer peripheral zone [all $F < 1$]).

Recurrent Hypoglycemia and Chronic Hyperglycemia Act Synergistically to Activate an Oxidative Stress Response

To explore the mechanisms underpinning the cognitive impairments demonstrated in working and long-term memory, transcript abundance of transcription factor Nrf2 as well as that of its target genes *Hmox1*, *Nqo1*, *Gsta1*, *Txn1*, *Txnrd1*, and *Srxn1* were measured in the hippocampus of each animal model. Significant changes in gene expression were seen in response to T1D and Hypo in both wild-type and $Nrf2^{-/-}$ animals (Table 1). Both T1D and T1D + Hypo increased mRNA for *Hmox1* and *Nqo1* in all animals, whereas a marked increase in Nrf2 expression was seen in the hippocampus of T1D + Hypo wild-type mice. As expected, the expression of other Nrf2-regulated genes (*Gsta1*, *Txn1*, *Txnrd1*, and *Srxn1*) appeared to be significantly diminished in all $Nrf2^{-/-}$ mice irrespective of the metabolic model applied. Interestingly, mRNA for *Gsta1* was significantly

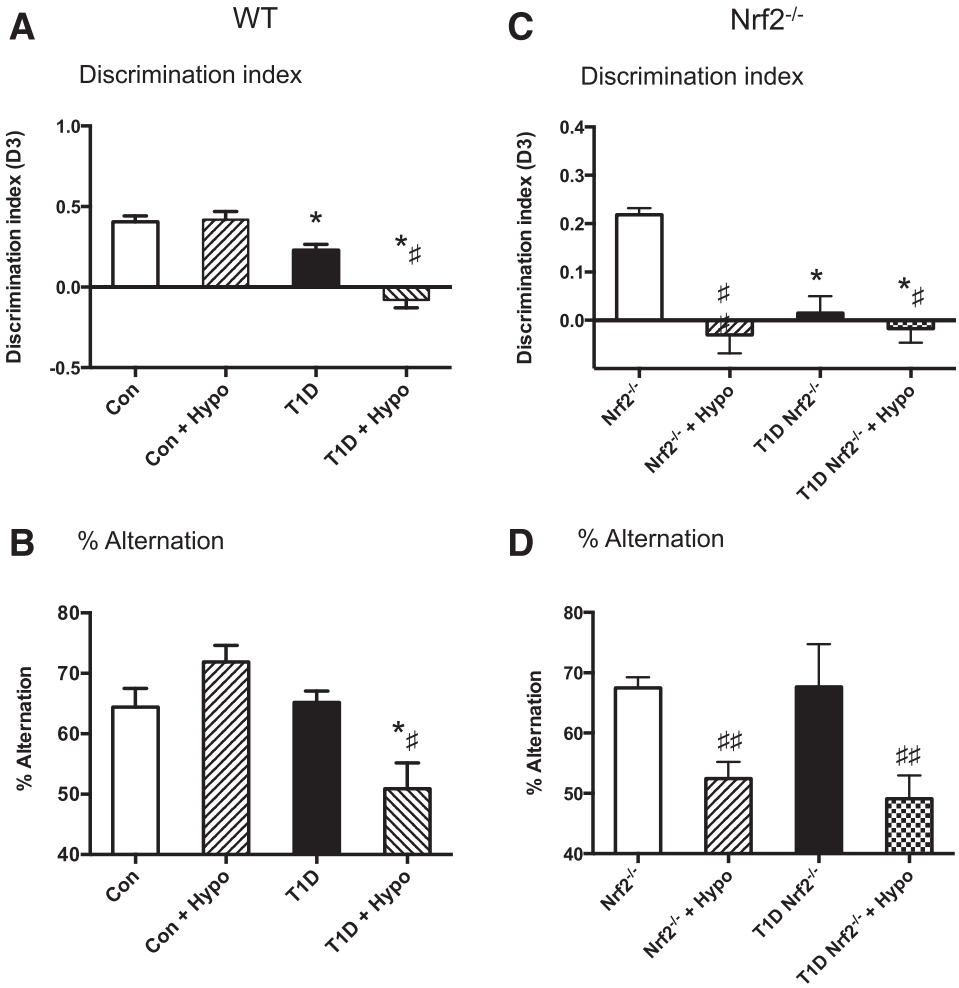

Figure 2—Recurrent hypoglycemia in T1D mice impairs cognitive performance in NOR and spontaneous alternation tasks. *A*: D3 demonstrating that T1D mice are significantly impaired when tested in the 24-h NOR task. This is exacerbated in T1D animals following Hypo. *B*: Mean 4/5 alternation performance on a closed arm plus maze, expressed as a percentage of possible alternations, is significantly reduced in T1D animals following Hypo. *C*: *Nrf2*$^{-/-}$ and T1D *Nrf2*$^{-/-}$ mice following Hypo were significantly impaired when tested in the 24-h NOR task. *D*: Mean 4/5 alternation performance on a closed arm plus maze, expressed as a percentage of possible alternations, is significantly reduced in *Nrf2*$^{-/-}$ + Hypo and T1D *Nrf2*$^{-/-}$ + Hypo animals when compared with their Control (No Hypo) counterparts. Control (Con), Con + Hypo, T1D, and T1D + Hypo, all n = 8/group. *Nrf2*$^{-/-}$, *Nrf2*$^{-/-}$ + Hypo, T1D *Nrf2*$^{-/-}$, and T1D *Nrf2*$^{-/-}$ + Hypo, all n = 7 to 8/group. Results represent mean values ± SEM. Data were analyzed by two-way ANOVA with T1D and Hypo as between subject factors followed by Tukey post hoc test. *$P < 0.05$ Control or *Nrf2*$^{-/-}$ vs. T1D or T1D *Nrf2*$^{-/-}$, #$P < 0.05$, ##$P < 0.01$ Hypo vs. No Hypo.

decreased in response to Hypo in T1D and nondiabetic WT mice, and this effect was lost in *Nrf2*$^{-/-}$ mice (Table 1).

Recurrent Hypoglycemia and Chronic Hyperglycemia Act Synergistically to Provoke ROS-Induced Cell Damage in the Hippocampus

To examine for evidence of ROS-induced cellular damage, levels of lipid peroxidation and protein carbonylation were determined in hippocampal homogenates. Hypo in both nondiabetic and T1D mice increased lipid peroxidation within the hippocampus (Fig. 3*A*) (Hypo, $F[1,30] = 8.36$; $P < 0.05$). Moreover, there was a T1D × Hypo interaction, indicating an additional stimulus to lipid peroxidation in T1D mice exposed to repeated hypoglycemia (T1D × Hypo, $F[1,28] = 6.24$; $P < 0.05$). Protein carbonylation, an irreversible process resulting from exposure to ROS and an indicator of severe oxidative damage (32), was significantly elevated in the hippocampus only in T1D mice exposed to Hypo (Fig. 3*B*) (T1D × Hypo, $F[1,28] = 4.35$; $P < 0.05$).

In *Nrf2*$^{-/-}$ mice, hippocampal lipid peroxidation (Fig. 3*C*) was increased by Hypo ($F[1,25] = 76.86$; $P < 0.01$), and greatest in T1D *Nrf2*$^{-/-}$ animals following Hypo (T1D × Hypo, $F[1,25] = 5.07$; $P < 0.05$). Protein carbonylation was increased with Hypo (Fig. 3*D*) (Hypo, $F[1,25] = 38.08$; $P < 0.01$), but not T1D ($F < 1$; P = not significant for T1D and T1D × Hypo).

Recurrent Hypoglycemia and Chronic Hyperglycemia Act Synergistically to Induce an Inflammatory Response Within the Hippocampus

To determine whether Hypo in T1D elicited an inflammatory response, cytokine protein levels were measured within hippocampal homogenates. In wild-type mice, IL-1β, IL-2, IL-4, IL-6, and TNF-α were significantly enhanced in T1D animals, whereas exposure to Hypo significantly

Table 1—Recurrent hypoglycemia and T1D in both wild-type and $Nrf2^{-/-}$ mice associated with modulation of genes involved in mediating antioxidant and redox systems

Gene	Control	Control + Hypo	T1D	T1D + Hypo	$Nrf2^{-/-}$	$Nrf2^{-/-}$ + Hypo	T1D $Nrf2^{-/-}$	T1D $Nrf2^{-/-}$ + Hypo
Hmox-1	1.00 ± 0.28	1.15 ± 0.14	1.34 ± 0.14*	1.88 ± 0.55*†	0.71 ± 0.07	0.49 ± 0.08	1.05 ± 0.11**	1.15 ± 0.11**†
Nrf2	1.00 ± 0.15	1.14 ± 0.18	1.86 ± 0.18*	12.13 ± 0.10*†	ND	ND	ND	ND
Nqo1	1.00 ± 0.35	1.53 ± 0.17	2.11 ± 0.28*	3.20 ± 0.44†	1.94 ± 0.13	1.78 ± 0.12	2.18 ± 0.31*	2.74 ± 0.24*†
Txnrd1	1.00 ± 0.19	1.07 ± 0.10	1.08 ± 0.10	1.09 ± 0.13	0.79 ± 0.05	0.95 ± 0.12	0.60 ± 0.08	0.59 ± 0.05
Srxn1	1.00 ± 0.09	0.94 ± 0.06	1.06 ± 0.03	1.13 ± 0.14	0.58 ± 0.10	0.71 ± 0.16	0.43 ± 0.05	0.48 ± 0.04
Txn1	1.00 ± 0.16	0.96 ± 0.11	1.28 ± 0.19	1.32 ± 0.23	0.82 ± 0.06	0.74 ± 0.11	0.73 ± 0.11	0.76 ± 0.11
Gsta1	1.00 ± 0.13	0.50 ± 0.76†	1.00 ± 0.06	0.53 ± 0.16†	0.11 ± 0.04	0.22 ± 0.07	0.10 ± 0.04	0.10 ± 0.04

mRNA was extracted and processed for real-time PCR to evaluate changes in gene expression of hemeoxygenase 1 (*Hmox-1*), *Nrf2*, NAD(P)H:quinoneoxidoreductase (*Nqo1*), thioredoxinreductase 1 (*Txnrd1*), sulfiredoxin 1 (*Srxn1*), thioredoxin 1 (*Txn1*), and glutathione S-transferase α subunit 1 (*Gsta1*). Control, Control + Hypo, T1D, and T1D + Hypo, all $n = 8$ group. $Nrf2^{-/-}$, $Nrf2^{-/-}$ + Hypo, T1D $Nrf2^{-/-}$, and T1D $Nrf2^{-/-}$ + Hypo, all $n = 7$ to 8/group. Results represent mean values ± SEM. Data were analyzed within genotype by two-way ANOVA with T1D and Hypo as between-subject factors followed by Tukey post hoc test. ND, not determined. *$P < 0.05$, **$P < 0.01$ Control or $Nrf2^{-/-}$ vs. T1D or T1D $Nrf2^{-/-}$. †$P < 0.05$, Hypo vs. No Hypo.

increased levels of IL-12p70 and IL-5 (Table 2) (all $P <$ 0.05). The combination of Hypo + T1D resulted in a marked stimulus to the production of IL-1β, IL-2, TNF-α, IL-4, IL-6, IL-12p70, and IL-5 (Table 2) (all $P < 0.01$).

A similar, but more marked pattern in cytokine release was seen in $Nrf2^{-/-}$ animals. IL-1β, IL-2, TNF-α, IL-4, IL-6, IL-12p70, and IL-5 levels were increased in T1D, whereas IL-6, IL-12p70, and IL-5 were significantly increased following Hypo (Table 2) (all $P < 0.05$). Again, in $Nrf2^{-/-}$ mice the combination of Hypo + T1D resulted in a marked stimulus to the production of IL-1β, IL-2, TNF-α, IL-4, IL-6, IL-12p70, and IL-5 (Table 2) (all $P <$ 0.001). Interestingly, levels of all cytokines measured were increased even in untreated $Nrf2^{-/-}$ mice and amplified following each metabolic stimulus, although the pattern of change was nearly identical to that seen in wild-type mice.

The transcript abundance of the inflammatory genes *IL-1β*, *IL-6*, *Tnfα*, and *Nos2* were also checked and showed a similar pattern of change to protein levels of each cytokine, although these increases only reached significance in T1D and T1D + Hypo $Nrf2^{-/-}$ animals (Supplementary Table 2).

DISCUSSION

In this study, we show for the first time in a healthy insulin-treated animal model of T1D that recurrent nonsevere hypoglycemia may impact on neuronal integrity and function. We show that eight episodes of nonsevere hypoglycemia in T1D over a 4-week period induces defects in memory consolidation and working memory, which is associated with persisting biochemical evidence of oxidative stress and inflammation in the hippocampus. Interestingly, protein synthesis, which is considered to play an integral role in memory consolidation, is disrupted at many levels by oxidative stress (33). In contrast and consistent with the work of others, recurrent hypoglycemia in nondiabetic rodents did not impair cognitive function (34). A key role for oxidative stress in inducing cognitive impairment was further illustrated by demonstrating a marked amplification of the hippocampal oxidative stress and inflammatory response in mice lacking the Nrf2 transcription factor (following recurrent hypoglycemia).

Preclinical and clinical studies suggest that marked glycemic variability maybe detrimental to humans (22,35–37). In the current study, glucose variability is represented by three principal metabolic states, namely chronic hyperglycemia, acute hypoglycemia, and recovery from acute hypoglycemia. Hypoglycemia can cause oxidative stress (20) and inflammation (21), and hypoglycemia-mediated ROS production can induce apoptosis (38,39). However, the fact that no impairment in cognitive performance was seen following Hypo in wild-type mice implies that the intrinsic antioxidant capacity, coupled with the oxidative stress response orchestrated by Nrf2, is ordinarily sufficient to protect the neuron from the consequences of moderate glucose deprivation. Consistent with this, recurrent hypoglycemia despite recovery to normal glucose levels resulted in cognitive impairment in $Nrf2^{-/-}$ mice. More recent studies in hippocampal slice

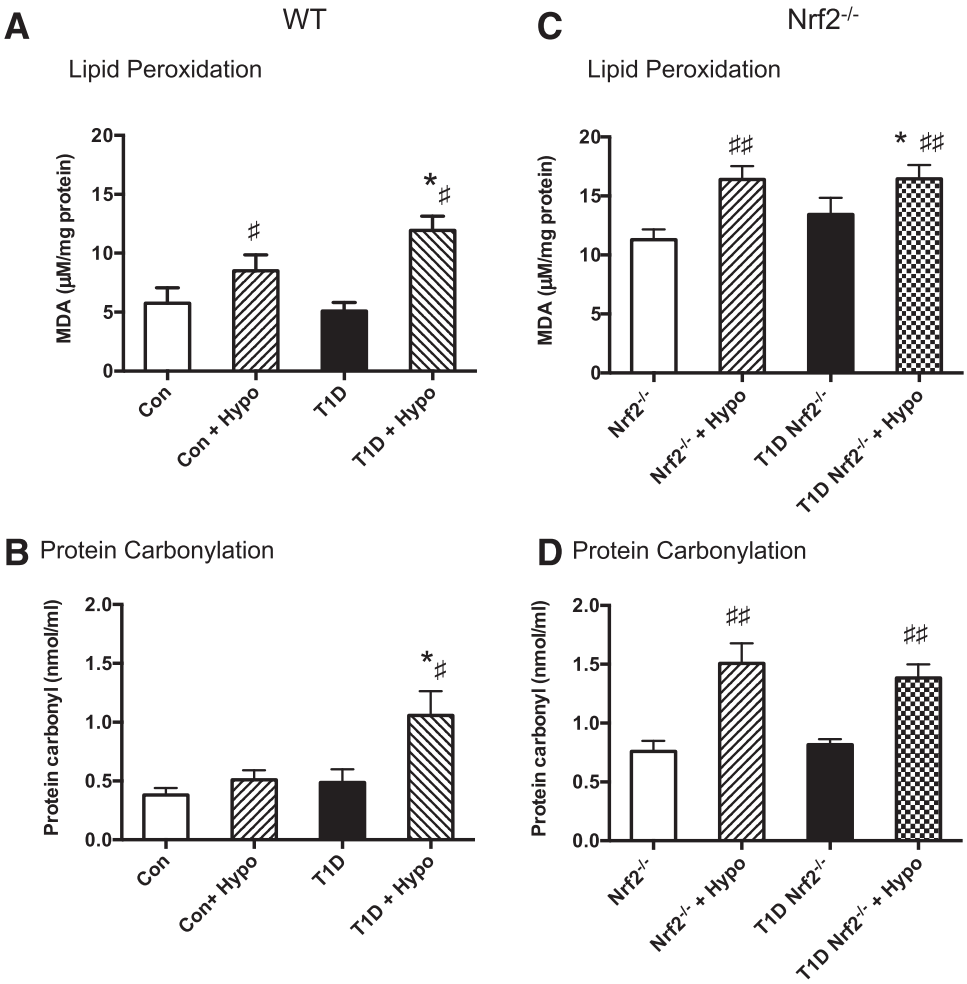

Figure 3—Recurrent hypoglycemia in T1D is associated with increased markers of oxidative damage. The levels of lipid peroxidation were determined using the thiobarbituric acid–reactive substances assay and protein carbonylation by ELISA. The levels of lipid peroxidation (*A*) and protein carbonylation (*B*) were significantly elevated following Hypo and further enhanced in T1D mice. The levels of lipid peroxidation (*C*) and protein carbonylation (*D*) were augmented following Hypo in *Nrf2*$^{-/-}$ mice and enhanced further in T1D *Nrf2*$^{-/-}$ + Hypo animals. Control (Con), Con + Hypo, T1D, and T1D + Hypo, all n = 8/group. *Nrf2*$^{-/-}$, *Nrf2*$^{-/-}$ + Hypo, T1D *Nrf2*$^{-/-}$, and T1D *Nrf2*$^{-/-}$ + Hypo, all n = 7 to 8/group. Results represent mean values ± SEM. Data were analyzed by two-way ANOVA with T1D and Hypo as between subject factors followed by Tukey post hoc test. *$P < 0.05$ Control or *Nrf2*$^{-/-}$ vs. T1D or T1D *Nrf2*$^{-/-}$, #$P < 0.05$, ##$P < 0.01$ Hypo vs. No Hypo. MDA, malondialdehyde.

preparations have shown that oxidative stress and neuronal death occur primarily in the recovery period from hypoglycemia during glucose reperfusion and that the extent of oxidative stress correlates with the rise in glucose during recovery (13). In our model, glucose levels post-hypoglycemia were >16 mmol/L, and therefore, glucose reperfusion into neurons that have experienced prolonged energy deprivation (as in ischemia reperfusion injury) may be the major contributor to oxidative stress. But Nrf2 protein levels and function are decreased in humans and rodents with diabetes (40,41); therefore, the Nrf2-mediated defense mechanism may also be insufficient to prevent oxidative damage resulting from nonsevere hypoglycemia in T1D. Neuronal damage secondary to severe hypoglycemia is exacerbated in T1D rats compared with nondiabetic Control subjects (14). Taken together, our data provide robust evidence that recurrent nonsevere hypoglycemia in T1D can provoke sufficient oxidative stress to induce a local inflammatory response and result in neuronal dysfunction, but cannot differentiate between potential effects of hypoglycemia per se or recovery to hyperglycemic levels. Future studies will be required to address this clinically important question.

Chronic hyperglycemia increases the production of ROS through mechanisms such as glucose auto-oxidation and nonenzymatic protein glycation (23) and lowers antioxidant defense mechanisms (24,25) as well as serum free-radical trapping capacity (23). In the current study, both T1D wild-type and T1D *Nrf2*$^{-/-}$ mice demonstrated impairments in memory consolidation. Neither model had tissue evidence of increased lipid peroxidation or protein carbonylation, suggesting no significant oxidative damage, but both models had significantly increased levels of *Nrf2*, *Hmox-1*, *Nqo-1*, and the inflammatory cytokines *IL-1β*, *IL-2*, *IL-4*, *IL-6*, and *Tnfα*. Intriguingly, loss of Nrf2 amplified both the inflammatory response and cognitive defect. It is recognized that inflammatory responses are exacerbated

Table 2—Recurrent hypoglycemia and T1D induce inflammation within the hippocampus in both WT T1D animals and *Nrf2*$^{-/-}$ animals, respectively

Protein (pg/mg)	Control	Control + Hypo	T1D	T1D + Hypo	Nrf2$^{-/-}$	Nrf2$^{-/-}$ + Hypo	T1D Nrf2$^{-/-}$	T1D Nrf2$^{-/-}$ + Hypo
IFN-γ	1.26 ± 0.19	1.01 ± 0.19	1.21 ± 0.19	1.16 ± 0.29	2.04 ± 0.13	1.99 ± 0.24	1.97 ± 0.21	1.73 ± 0.30
KC/GRO	57.60 ± 7.42	68.86 ± 10.73	65.15 ± 9.51	59.57 ± 7.03	99.49 ± 7.24	95.35 ± 12.47	102.7 ± 12.12	116.7 ± 21.47
IL-1β	1.63 ± 0.14	1.72 ± 0.26	2.68 ± 0.25**	3.15 ± 0.43**†	2.96 ± 0.49	2.62 ± 0.39	6.95 ± 0.80*	9.40 ± 1.23*†
IL-2	5.20 ± 1.10	6.19 ± 0.93	6.80 ± 1.27*	10.72 ± 1.23*†	6.47 ± 0.76	7.10 ± 1.10	10.91 ± 1.15**	10.88 ± 0.86*†
TNF-α	1.87 ± 0.45	1.39 ± 0.73	4.28 ± 0.44*	7.37 ± 0.61*†	4.97 ± 0.86	7.34 ± 1.40	10.41 ± 2.28*	10.75 ± 1.98*†
IL-4	1.56 ± 0.26	1.32 ± 0.26	3.05 ± 0.52**	4.35 ± 0.34*†	2.16 ± 0.32	3.28 ± 0.53	4.96 ± 0.58**	5.34 ± 0.57*†
IL-6	85.54 ± 13.29	106.4 ± 16.11	146.8 ± 17.30**	178.3 ± 9.32*†	99.48 ± 11.59	140.4 ± 3.99†	145.4 ± 8.58**	149.6 ± 6.88*†
IL-12p70	20.20 ± 1.31	25.84 ± 1.15†	24.83 ± 1.71	27.65 ± 02.09*†	20.00 ± 3.25	27.63 ± 2.83††	22.02 ± 3.84*	35.23 ± 4.27*††
IL-5	5.39 ± 0.68	7.17 ± 0.41†	5.11 ± 0.69	8.48 ± 0.74*††	5.48 ± 0.66	6.78 ± 0.31†	8.45 ± 0.59**	7.58 ± 0.22*†

Levels of a number of common inflammatory cytokines were measured within hippocampal homogenates by ELISA. Control, Control + Hypo, T1D, and T1D + Hypo, all n = 8/group. *Nrf2*$^{-/-}$, *Nrf2*$^{-/-}$ + Hypo, T1D *Nrf2*$^{-/-}$, and T1D *Nrf2*$^{-/-}$ + Hypo, all n = 7 to 8/group. Results represent mean values ± SEM. Data were analyzed within genotype by two-way ANOVA with T1D and Hypo as between-subject factors followed by Tukey post hoc test. GRO, human growth-related oncogene; KC, keratinocyte chemoattractant. *$P < 0.05$, **$P < 0.01$ Control or *Nrf2*$^{-/-}$ vs. T1D or T1D *Nrf2*$^{-/-}$. †$P < 0.05$, ††$P < 0.01$ Hypo vs. No Hypo.

in *Nrf2*$^{-/-}$ mice (42,43), and such an outcome is consistent in humans and rodents with diabetes in which Nrf2 levels and activity are diminished (40,41). This is consistent with a recognized hierarchical response to oxidative stressors by which modest levels of ROS activate an Nrf2-orchestrated adaptation, whereas higher levels of ROS stimulate NF-κB and AP-1 to provide an additional defense mechanism (44). In this case, *Nrf2*$^{-/-}$ mice would be anticipated to have higher levels of ROS under both hypo- and also hyperglycemia conditions, leading to a proinflammatory state. Recent studies indicating a role for Nrf2 agonists in the treatment of diabetic nephropathy (43) and cardiomyopathy (45) that are associated with chronic hyperglycemia would be consistent with this possibility. The results of the current study would suggest that chronic hyperglycemia induces a proinflammatory condition through Nrf2-dependent and -independent mechanisms.

A critical and novel finding in this study is the important role of the transcription factor Nrf2 in initiating the oxidative stress response to hypoglycemia. Nrf2 is a transcription factor that dictates the intrinsic antioxidant capacity of cells under normal physiological conditions and also directs adaptation to oxidative stress. The activity of Nrf2 is itself regulated through a complex transcriptional/epigenetic and posttranslational network in a manner that ensures its function increases during redox perturbation, inflammation, and nutrient/energy fluxes, thereby enabling the factor to orchestrate adaptive responses to diverse forms of stress (for a review, see Ref. 26). Previously, Kraft et al. (46) have reported that *Nrf2*$^{-/-}$ mice are significantly more sensitive to kainate neuronal toxicity than their wild-type counterparts and used microarray analysis to show this was associated with markedly reduced expression in the hippocampus of *aldehyde oxidase 1*, *Gstm1*, *Gstm3*, *peroxiredoxin 1* (*Prdx1*), and *Prdx2*. In our study, the responses of *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice to T1D and Hypo were examined in separate experiments, and we therefore cannot strictly compare gene expression profiles between wild-type and knockout mice. Nevertheless, it was apparent from our gene expression analyses that large differences exist between the expression of *Gsta1*, *Gstm1*, and *Srxn1*in the hippocampus of in *Nrf2*$^{+/+}$ and *Nrf2*$^{-/-}$ mice. In the future, it will be desirable to examine simultaneously the influence that loss of Nrf2 and genetic upregulation of Nrf2 (caused by diminished expression of Kelch-like ECH-associated protein 1 [Keap1], a negative regulator of Nrf2) has on gene expression and cognitive function following T1D and Hypo. It is notable that we found the levels of mRNA for Nrf2 were significantly upregulated by T1D and very dramatically (by 12-fold) by T1D + Hypo. These results are consistent with a recent report that the protein levels of Nrf2 are increased in the diabetic wounds of humans and mice and that pharmacological activation of Nrf2 promotes wound healing in T1D mice (47). Together with the heightened inflammation in T1D and T1D *Nrf2*$^{-/-}$ + Hypo mice relative to their wild-type counterparts, these findings suggest that a function of Nrf2 is to act as a brake to control inflammation. Our

current results illustrate the critical importance of this function of Nrf2: indeed, inflammation is controlled, and cognitive function is largely preserved in wild-type animals subjected to hypoglycemia, whereas inflammation is enhanced and cognitive function is severely impaired in their $Nrf2^{-/-}$ counterparts.

In conclusion, our study supports the hypothesis that recurrent moderate hypoglycemia in T1D may have long-term consequences on cognitive function. Our findings suggest that chronic hyperglycemia, recurrent hypoglycemia, and glucose reperfusion following hypoglycemia in T1D interact synergistically to induce pathological oxidative stress in vulnerable brain regions such as the hippocampus. Whether hypoglycemia per se or glucose recovery from hypoglycemia is the major contributor to oxidative damage in T1D cannot be determined from our studies although preclinical research suggests the recovery period is key. This has implications for clinical practice in which treatment of hypoglycemia often leads to marked rebound hyperglycemia. Moreover, we provide evidence that the transcription factor Nrf2 may be integral to neuronal protection against oxidative stress during and following hypoglycemia and in response to chronic hyperglycemia. This raises the possibility of targeting Nrf2 in developing therapies designed to prevent cellular damage in diabetes induced by recurrent hypoglycemia.

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