Thyroxin Treatment Protects Against White Matter Injury in The Immature Brain via Brain-Derived Neurotrophic Factor

Pi-Lien Hung, MD; Chao-Ching Huang, MD; Hsiu-Mei Huang, MD; Dom-Gene Tu, MD; Ying-Chao Chang, MD, PhD

Background and Purpose—Low level of thyroid hormone is a strong independent risk factor for white matter (WM) injury, a major cause of cerebral palsy, in preterm infants. Thyroxin upregulates brain-derived neurotrophic factor during development. We hypothesized that thyroxin protected against preoligodendrocyte apoptosis and WM injury in the immature brain via upregulation of brain-derived neurotrophic factor.

Methods—Postpartum (P) day-7 male rat pups were exposed to hypoxic ischemia (HI) and intraperitoneally injected with thyroxin (T4; 0.2 mg/kg or 1 mg/kg) or normal saline immediately after HI at P9 and P11. WM damage was analyzed for myelin formation, axonal injury, astrogliosis, and preoligodendrocyte apoptosis. Neurotrophic factor expression was assessed by real-time polymerase chain reaction and immunohistochemistry. Neuromotor functions were measured using open-field locomotion (P11 and P21), inclined plane climbing (P11), and beam walking (P21). Intracerebroventricular injection of TrkB-Fc or systemic administration of 7,8-dihydroxylavone was performed.

Results—On P11, the HI group had significantly lower blood T4 levels than the controls. The HI group showed ventriculomegaly and marked reduction of myelin basic protein immunoreactivities in the WM. T4 (1 mg/kg) treatment after HI markedly attenuated axonal injury, astrogliosis, and microgliosis, and increased preoligodendrocyte survival. In addition, T4 treatment significantly increased myelination and selectively upregulated brain-derived neurotrophic factor expression in the WM, and improved neuromotor deficits after HI. The protective effect of T4 on WM myelination and neuromotor performance after HI was significantly attenuated by TrkB-Fc. Systemic 7,8-dihydroxylavone treatment ameliorated hypomyelination after HI injury.

Conclusions—T4 protects against WM injury at both pathological and functional levels via upregulation of brain-derived neurotrophic factor—TrkB signaling in the immature brain. (Stroke. 2013;44:2275-2283.)

Key Words: brain-derived neurotrophic factor ■ hypoxic ischemia ■ immature brain ■ thyroxin ■ white matter injury

Periventricular white matter (WM) injury is a major cause of brain injury and accounts for the most prominent determinant of neuromotor deficits in preterm infants. Although the pathogenesis is multifactorial, hypoxic ischemia (HI) is the major risk factor. Human and animal studies demonstrate that HI injury in preterm infants particularly targets the oligodendrocyte lineage, whereas neuronal and glial cells are markedly more resistant. The timing of appearance of preoligodendrocyte lineage, whereas neuronal and glial cells are markedly more resistant.1 The timing of appearance of preoligodendrocyte lineage, whereas neuronal and glial cells are markedly more resistant.

Thyroid hormone (TH) is essential for oligodendrocyte maturation and myelination. It acts via multiple steps in the development of oligodendrocytes. TH enhances the proliferation of the committed preprecursor oligodendrocytes in early development and increases morphological and functional maturation in postmitotic oligodendrocytes. Myelination is delayed in hypothyroid animals but accelerated in hyperthyroid animals. Low levels of TH are commonly found in the first weeks after birth in preterm babies, a phenomenon called transient hypothyroxinemia of prematurity. Transient hypothyroxinemia of prematurity is a strong independent risk factor for WM injury, cerebral palsy, and lower cognitive performance in preterm infants. However, it remains unknown whether exogenous administration of
thyroxin might stimulate the repair potential of endogenous pre-OLs and protect against WM injury in the immature brain. Thyroxin acts through nuclear TH receptors as ligand-regulated transcription factors. Thyroxin regulates neurotrophic gene expression in a developmental stage- and brain region-specific manner. Early postnatal thyroxin treatment causes upregulation of brain-derived neurotrophic factor (BDNF) mRNA expression in the hippocampus. BDNF mRNA and protein are significantly increased in the medial septum on postpartum (P) day-10 rats after transient thyroxin treatment. Here, we hypothesized that thyroxin treatment protected against pre-OLs and WM injury in the immature brain via upregulating BDNF.

**Methods**

**WM Injury**

This study was approved by the Chang Gung Memorial Hospital Committee on the Use and Care of Animals. The P7 male rat pups were anesthetized with 2.5% halothane (balance, room air), and the right common carotid artery was permanently ligated. After surgery, the pups had a 1-hour recovery before being placed in airtight 500-mL airstight containers in a 37°C water bath through which humidified 3 L/min 6.5% oxygen was maintained for 1 hour. For thyroxin treatment trials, pups were randomized to receive intraperitoneal injection of L-thyroxin (Sigma) 0.2 mg/kg (T4−0.2 group; n=26), 1 mg/kg (T4−1 group; n=28), or normal saline (NS group; n=25) immediately after hypoxia, and at P9 and P11. For BDNF treatment, pups were randomized to receive intraperitoneal injection of 7,8-dihydroxyflavone (Sigma), a small-molecule imitating BDNF, 5 mg/kg (n=12) or dimethyl sulfoxide (n=10) 2 hours before ligation, and at P9 and P11. The control group received no HI or any injection. Blood samples were obtained from pups on P7 (immediately after hypoxia), P11, and P21. T4 levels were determined by radioimmunoassay (Siemens). The biochemical and behavioral measurements were performed by the investigators who were blinded to the treatment grouping during the assessment.

**Intracerebral Injections**

TrkB-Fc (1.5 μg in 6 μL; Sigma) or phosphate buffered saline was given intracerebroventricularly 3 hours after each T4 administration on P7, P9, and P11. Intracerebroventricular injection in the right cerebral hemisphere was performed (in relation to the bregma: 1.0 mm posterior to, 1.0 mm lateral to, and 1.0 mm beneath the skull surface) after the rats were anesthetized intraperitoneally by 10% atropine and 6% chloral hydrate.

**Outcome Evaluation**

**Neuromotor Assessment**

Neuromotor assessments were performed using open-field locomotion (P11 and P21), inclined plane climbing (P11), and beam walking (P21). Open-field locomotion was scored: 0, cannot walk; 1, left leg limping; 2, normal gait. The climbing behaviors at different angles of inclined plane were scored: 1, hanging on; 2, turning and going down; 3, walking up without sliding down; and 4, running up. Beam walking through the entire length of a standard beam to the goal box within 60 seconds was scored: 0, incapable of performing task; 1, foot drop; and 2, normal.

**Histological Analysis**

On P11, the paraffin-embedded brain was sectioned coronally (6 μm thick). Four sections per brain, 2 at the level of the striatum (0.26 mm and 0.92 mm posterior to the bregma) and 2 at the dorsal hippocampus levels (3.14 mm and 4.16 mm posterior to the bregma), were selected for immunohistochemical staining. Brain damage was evaluated using hematoxylin-eosin and Fluoro-Jade C staining. The means of the ratio of ventricular size (R/L) for those sections were obtained.

**Immunofluorescence**

The sections were incubated with the primary antibodies: anti-CD68 antibody (ED1, 1:200; Serotec), antiligilary acidic protein (GFAP, 1:200; Dako), anti-O1 (1:200; Chemicon), and anti-O4 (1:200; Chemicon), followed by Alexa Fluor 594 antimouse IgG/IgM or Alexa Fluor 488 anti-rabbit IgG (1:400; Invitrogen), and photographed for red (Alexa Fluor 594) and green (Alexa Fluor 488) fluorescence using a fluorescent microscope (E400; Nikon).

For double labeling, apoptotic cells were stained with terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) using an ApopTag fluorescent direct in situ apoptosis detection kit (Chemicon). The sections were then incubated with anti-O4 antibody, followed by Alexa Fluor 488 antibody. The number of TUNEL(+)O4(+) cells was counted in an area of 40 μm × 100 μm at 400 magnification, using a fluorescence microscope. For labeling the proliferating pre-OLs, sections were incubated with both anti–Ki-67 (1:100; Abcam) and anti-O4 primary antibodies, followed by Alexa Fluor 488 and 594 antibodies. The number of proliferating pre-OLs was counted in an area of 200 μm × 200 μm at 200 magnification.

**Assessment of Immunohistochemical Staining**

Measurement of O1-, O4-, GFAP-, ED1-, Ki-67-, and TUNEL-positive cells, and of the integrated OD (IOD) of β-APP, MBP, BDNF signals were respectively analyzed as described previously using an imaging software (ImagePro Plus 6.0) at 200 magnification for MBP, GFAP, Ki-67, β-APP, and ED1 scores and at 400 magnification for O1, O4, and TUNEL signals. Three visual fields in the medial, middle, and lateral areas of WM in each hemisphere per section, and 4 sections per brain as described previously, were analyzed and averaged, respectively. The mean IOD values in the ipsilateral WM of each experimental group were compared with those of the control group to obtain the relative IOD ratios.

**Real-Time Polymerase Chain Reaction**

Total RNA was extracted and real-time polymerase chain reaction performed for BDNF, neurotrophic growth factor (NGF), insulin-like growth factor-1, and platelet-derived growth factor–α3 (see the Methods section in the online-only Data Supplement).

**Statistical Analysis**

Continuous data were mean±standard errors of mean (SEM). Statistical significance (P<0.05) was determined using 1-way
ANOVA, whereas the Tukey method was used for post hoc comparisons.

**Results**

**HI Caused Hypothyroixemia and WM Injury in Immature Brain**

Compared with the control group, the HI group had similar mean blood levels of T4 immediately after HI on P7, but showed significantly decreased T4 levels on P11 ($P<0.05$; Figure 1A). Hematoxylin-eosin staining on P11 showed no obvious damage in the ipsilateral cortex except a few eosinophilic neurons in the HI group (Figure 1B). Instead, the HI group had ventriculomegaly and marked reduction of MBP immunoreactivities in the WM of the ipsilateral hemisphere (Figure 1C).

**T4 Decreased Astrocytosis and Microgliosis and Protected Against Pre-OLs Apoptosis in the WM After HI**

After treatment, the control, NS, T4–1, and T4–0.2 groups had similar body temperature and body weight from P7 to P21. The T4–1 but not the T4–0.2 group had significantly more O1(+) and O4(+) cells than the NS group (both $P<0.05$; Figure 2B). The NS group also had significantly more activated microglia that showed large round cell bodies and reduced processes in the WM. The T4–1 but not T4–0.2 group had significantly lower ED1(+) microglia than the NS group ($P<0.01$).

On P11, immunofluorescence revealed that the NS group had significantly more TUNEL(+)-O4(+) cells in the WM than the control group ($P<0.001$; Figure 2C). The T4–1 but not T4–0.2 group showed significantly less TUNEL(+)-O4(+) cells than the NS group ($P<0.001$). Double labeling using Ki-67 and O4 showed that the NS group had significantly more proliferating pre-OLs than the control group ($P<0.01$), whereas the Tukey method was used for post hoc comparisons.

**Figure 1.** Hypoxic ischemia (HI) caused hypothyroixemia and white matter (WM) injury in immature brain. **A**, Significantly decreased blood levels of T4 were noted in P11 pups but not in P7 pups. **B**, Hematoxylin-eosin (HE) staining showed a few eosinophilic neurons (arrows) in the ipsilateral (#) cortex. **C**, MBP immunostaining showed marked ventriculomegaly and reduced myelin basic protein (MBP) immunoreactivity in the ipsilateral white matter after HI injury. Scale bar=200 μm in B; 50 μm in B insets and 100 μm in C insets. *$P<0.05$.**
whereas the T4−1 group showed significantly lower Ki67(+) O4(+) cells than the NS group (P<0.001; Figure 2D).

**T4 Selectively Upregulated BDNF in the WM After HI**

On P11, there were no significant differences in NGF, insulin-like growth factor-1, and platelet-derived growth factor-α mRNA expression in the WM among the NS- and T4-treated HI groups. In contrast, the T4−1 group had significantly increased BDNF mRNA expression than the NS group (P≤0.05; Figure 3A). The NS- and T4-treated HI groups had similar NGF and BDNF mRNA expression in the cortex (Figure I in the online-only Data Supplement). Immunostaining revealed that the 4 groups showed comparable BDNF protein levels in the cortex (data not shown). The NS group had significantly decreased BDNF expression in the WM (P<0.001), whereas the T4−1 but not T4−0.2 group had markedly higher BDNF expression than the NS group (P<0.01; Figure 3B and 3C).

**T4 Attenuated WM Injury and Improved Neuroromotor Deficits After HI**

On P11, both NS- and T4-treated groups showed a few eosinophilic cells (hematoxylin–eosin; Figure 4A) and degenerated
neurons (Fluoro-Jade C; Figure 4B) in the cortex. However, there was significant ventricular dilatation in the NS groups (P<0.01). The T4−1 group had significantly lower ventriculomegaly than the NS group (P<0.01; Figure 4A). The NS group had prominent β-APP immunoreactivity (P<0.005; Figure 4C), suggesting axonal injury, and significant reduction of MBP immunoreactivities (P<0.005; Figure 4D), suggesting hypomyelination in the WM than the control group. The T4−1 group had significantly lower β-APP (P<0.005) and higher MBP (P<0.005) immunostaining in the WM than the NS group.

Gait analysis showed that compared with the control group, the NS group had significant contralateral limb dysfunction on P11 (P<0.01) and P21 (P<0.001). The T4−1 but not the T4−0.2 group had significantly better gait performance than the NS group on P11 (P<0.005) and P21 (P<0.001). On P11, the NS group also performed significantly worse than the control group (P<0.05) on the 45° inclined plane, whereas the T4−1 group performed significantly better than the control group (P<0.05). There were no differences in the performance of gait analysis and inclined plane between the control and T4−1 group. Beam-walking test on P21 revealed that the NS and T4−0.2 groups had significantly more frequent left foot drop than the control group (P<0.001; Figure 5A). The T4−1 group had significantly better scores than the NS group (P<0.01; Figure 5A).

TrkB-Fc Intracerebral Injection Attenuated the Protective Effect of T4 on Myelination and Neuromotor Outcome

The T4−1 pups were subjected to intracerebral infusion of TrkB-Fc, a scavenger of BDNF, or vehicle. Immunohistochemical studies demonstrated that the TrkB-Fc–treated group had significantly more MBP damage in the WM than the vehicle-treated group (P<0.001; Figure 5B). The vehicle-treated and the control group had comparable performance on gait analysis (P11) and beam walking (P21). In contrast, the TrkB-Fc–treated group had significantly worse performance by gait analysis (P<0.005) and beam walking (P<0.001) than the vehicle-treated group (Figure 5C).

Systemic TrkB Agonist Treatment Ameliorated WM Injury After HI

There was no difference in body temperature and body-weight gain between the 7,8-dihydroxyflavone–, dimethyl sulfoxide–treated HI rats and controls. The 7,8-dihydroxyflavone–treated group had significantly increased MBP immunodensity compared with the dimethyl sulfoxide–treated group (Figure 5D).

Discussion

The present study reveals that HI caused transient hypothyroidism and induced pre-OLs apoptosis and WM injury in the immature brain. T4 treatment after HI decreased axonal injury and astrogliosis, enhanced pre-OLs survival and myelination, protected against WM damage, and improved neuromotor deficits. T4 treatment also reduced the number of proliferating pre-OLs in the WM but favored pre-OLs survival via decreasing apoptotic cell death after HI. Moreover, T4 treatment selectively upregulated BDNF expressions in the WM, and inhibition of BDNF reversed the protective effect of T4 at pathological and neuromotor levels. TrkB activation enhanced myelination in WM after HI injury. Taken together, our study suggests that T4-mediated BDNF-TrkB signaling plays an important role in protecting against pre-OLs apoptosis and WM injury after HI in the immature brain.

Molecular, cellular, and functional analyses all support a transient role for TH during brain and oligodendrocyte development. TH plays at least 2 distinct roles in oligodendrocyte development: control of precursor cell proliferation and regulation of myelin gene expression. The pre-OLs responses after HI include cell death, survival with
loss of cell processes, and replenishment by proliferation. However, surviving pre-OLs may not differentiate to form mature, myelin-producing OLs, resulting in deficiency in mature OLs and cerebral hypomyelination.\textsuperscript{1} We found that although T4 post-treatment reduced the number of proliferating pre-OLs, the enhanced pre-OLs maturation and myelination may rescue WM from HI at both pathological and functional levels in the immature brain.

The present study provides the first in vivo evidence for the prosurvival effects of T4 on pre-OLs. Studies have shown increased neuronal apoptosis in the cerebellum and cortex of hypothyroid rat pups.\textsuperscript{16,17} In vitro studies showed that triiodothyronine rescued developing oligodendrocytes from death in a mixed cell but not pure oligodendrocytes culture.\textsuperscript{15,18} These findings suggested that TH may not directly act on the oligodendrocyte to ensure its survival but rather regulates the expression of growth factors that are required for pre-OLs survival. The potential growth factors required for oligodendrocyte survival include NGF, insulin-like growth factor-1, and platelet-derived growth factor–α.\textsuperscript{15} Our data demonstrated that T4-BDNF axis plays an important role in protecting pre-OLs against apoptosis.

TH regulates neurotrophic gene expression in a promoter-, developmental stage-, and brain region–specific manner.\textsuperscript{7} Perinatal hypothyroidism repressed the expression of BDNF in the hippocampus and cerebellum.\textsuperscript{19,20} Early postnatal T4 treatment causes upregulation of BDNF and neurotrophin-3, but not NGF, mRNA expression in the hippocampus.\textsuperscript{7} BDNF

**Figure 4.** T4 attenuated hypomyelination, axonal injury, and ventriculomegaly after hypoxic ischemia (HI). Hematoxylin-eosin (HE; A) and Fluoro-Jade C (FJC; B) stain showed a few eosinophilic (arrowheads) and degenerated neurons (arrows), respectively, in the cortex of both normal saline (NS)– and T4-treated groups. However, there was prominent ventricular dilatation in the NS groups. The T4–1 group had significantly less ventriculomegaly than the NS group. The NS group had significantly higher β-amyloid precursor protein (APP; C) and myelin basic protein (MBP; D) immunostaining in the white matter (WM) than the controls. The T4-treated groups had lower β-APP expression than the NS groups. The T4–1 but not T4–0.2 group had significantly more myelination in the WM than the NS group. Scale bar=200 μm in A and D, 50 μm in B, 100 μm in C, 50 μm in insets; *P<0.05; **P<0.01; ***P<0.005.
mRNA and protein are significantly increased in the medial septum on P10 but not adult rats after transient thyroxin treatment. Our data demonstrate that in the WM, T4 treatment after HI upregulates BDNF, but not NGF, insulin-like growth factor-1, and platelet-derived growth factor-α mRNA expression. Furthermore, intracerebral infusion of a BDNF scavenger significantly attenuates the protective effects of T4 treatment at both pathological and functional levels. Systemic administration of 7,8-dihydroxyflavone, a recently identified small-molecule TrkB agonist that can pass the blood–brain barrier, thus ameliorating hypomyelination after HI injury. These findings suggest that BDNF-TrkB signaling plays a necessary role for the region-specific protective effects of T4.

The underlying mechanism of T4-mediated reduced inflammation and axonal injury in the WM after HI remains to be determined. Selective decreases of microglial activation in the WM after T4 treatment corresponded to the region-specific decreases of pre-OLs and axonal injury. Activated microglia are the hallmark of neuron inflammation and exacerbate WM injury through the production of injurious cytokines and perturbations of glutamate homeostasis. Recent data identify T4 as a potential endogenous inhibitor of proinflammatory cytokines, and T4 administration after

Figure 5. T4–brain-derived neurotrophic factor (BDNF)–TrkB signaling attenuated white matter (WM) injury and improved neuromotor deficits after hypoxic ischemia (HI). A, Compared with the control group, the normal saline (NS) group had significant contralateral limb dysfunction on P11 and P21 on gait analysis. The T4–1 but not the T4–0.2 group had significantly better gait performance than the NS group. On P11, the NS group also performed significantly worse than the control group, whereas the T4–1 group performed significantly better than the NS group. Beam-walking test on P21 revealed that the NS and T4–0.2 groups had significantly more frequent left foot drop than the control group. The T4–1 group had significantly better scores than the NS group. In the T4-treated HI rats, TrkB-Fc treatment caused significantly more myelin basic protein (MBP) damage in the WM (B) and more neuromotor deficits (gait analysis on P11 and beam walking P21; C) than the vehicle group. D, After HI, the 7, 8-dihydroxyflavone (DHF)–treated group had significantly more MBP immunodensity in the WM than the dimethyl sulfoxide (DMSO)–treated group. Scale bar=100 μm in B and D. *P<0.05; **P<0.01; ***P<0.005; #P<0.001.
sepsis significantly reduces leukocyte accumulation and improves survival in mice.21 Thus, further study is needed to examine whether T4 treatment decreases proinflammatory cytokines, which contributes to the rescue of pre-OLs death and axonal injury in the WM. Although transient hypothyroxinemia of prematurity is a strong independent risk factor for cerebral palsy and cognitive delay,5,6 several T4-supplementation trials conducted in premature infants have shown inconclusive results.22,23 One potential explanation is related to the variety of dosage regimens.23 We showed that the protective effect of T4 is doserelated: effect occurs only in the T4−1 but not in the T4−0.2 group. The optimal dose for potential human treatment remains to be determined.

Summary
We found that T4 treatment protects against WM damage at both the pathological and functional levels via BDNF in the immature brain. Our findings highlight the potential role of T4 treatment against WM injury in preterm babies. A proposed schema is provided to show the T4-BDNF-mediated protective mechanisms in the WM after HI in the immature brain, enhancing myelination and pre-OLs survival and attenuating axonal injury and neuroinflammation (Figure 6).

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Disclosures
None.

References


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Supplemental Methods

Quantitative real-time PCR. The quantity and purity of extracted RNA (RNase Mini Kit; Qiagen) were estimated by measuring $A_{260}$ and $A_{280}$. cDNA was synthesized with MMLV reverse transcriptase (Promega). Real-time PCR (LC480 LightCycler Real-Time PCR System and software; Roche Diagnostics GmbH) was done using a 20-µl reaction mixture that contained 10-µl of SYBR Green (Roche), 4-µL of cDNA, and 1µM of primers [brain-derived neurotrophic factor (BDNF): AGCTTCATTCTGAGAGACG and GTCAACATAAACCACCGACA-3'; neurotrophic growth factor (NGF): TCTGAGGTGCATAGCGTAA and GGCTGTGTCAAGGGAAT; insulin-like growth factor (IGF-1): AAGCCTACAAAGTCAGCTCG and GGTCTTGTTTCCTGCACTTC; platelet derived growth factor α (PDGF α): CCTGTGCCCATCCGCAGGAA and TTGGCCACCTTGACGCTGC; β-actin: TACTGCCCTGGCTCCTA and GGGCCGGACTCATCGTA], and run in the Light Cycler real-time detection system (Roche). The hot-start enzyme was activated (95°C for 5 minutes) and cDNA was then amplified for 40 cycles consisting of denaturation at 95°C for 30 seconds and annealing/extension at 60°C for 30 seconds. A melt curve was then performed after amplification. β-actin used as an internal control was amplified with the primers. Amplicon size and reaction specificity were confirmed using 1% agarose gel electrophoresis. Data were analyzed using the Light Cycler software (Roche Diagnostics GmbH). The Ct value of fluorescence units was used for analysis, and quantification done using the Ct of target genes cDNA relative to that of β-actin -actin cDNA in the same sample.
Supplemental Figure S1

T4 selectively upregulated BDNF in the WM but not the cortex after HI. By RT-PCR, there was no significant differences in the NGF and BDNF mRNA expression in the ipsilateral cortex between and NS- and T4-treated groups.