The Effects of Repeat Traumatic Brain Injury on the Pituitary in Adolescent Rats

Tiffany Greco,^{1,2} David Hovda,^{1–4} and Mayumi Prins^{1–3}

Abstract

Adolescents are one of the highest groups at risk for sustaining both traumatic brain injury (TBI) and repeat TBI (RTBI). Consequences of endocrine dysfunction following TBI have been routinely explored in adults, but studies in adolescents are limited, and show an incidence rate of endocrine dysfunction in 16–61% in patients, 1–5 years after injury. Similar to in adults, the most commonly affected axis is growth hormone (GH) and insulin-like growth hormone 1 (IGF-1). Despite TBI being the primary cause of morbidity and mortality among the pediatric population, there are currently no experimental studies specifically addressing the occurrence of pituitary dysfunction in adolescents. The present study investigated whether a sham, single injury or four repeat injuries (24 h interval) delivered to adolescent rats resulted in disruption of the GH/IGF-1 axis. Circulating levels of basal GH and IGF-1 were measured at baseline, 24 h, 72 h, 1 week, and 1 month after injury, and vascular permeability of the pituitary gland was quantified via Evans Blue dye extravasation. Changes in weight and length of animals were measured as a potential consequence of GH and IGF-1 disruption. The results from the current study demonstrate that RTBI results in significant acute and chronic decreases in circulation of GH and IGF-1, reduction in weight gain and growth, and an increase in Evans Blue dye extravasation in the pituitary compared with sham and single injury animals. RTBI causes significant disruption of the GH/IGF-1 axis that may ultimately affect normal cognitive and physical development during adolescence.

Key words: adolescent; GH; mild TBI; pituitary; RTBI

Introduction

PITUITARY DYSFUNCTION has routinely been described following traumatic brain injury (TBI) in *adults* with deficiencies of growth hormone (GH) and gonadal axes being the most commonly occurring.^{1,2} TBI patients routinely have a host of complications that involve cognitive, physical, metabolic, and emotional abilities. Pituitary dysfunction is increasingly being recognized following TBI, and could play a major role in these complications. GH deficiency has been associated with more severe deficits in memory, attention, executive function, mood, and sleep, as well as being associated with fatigue, many of which are often reported as postconcussive symptoms.^{3–10} GH also may play a critical role in recovery, as it is involved in both myelin formation^{11–13} and neuronal plasticity.^{14–17}

A number of potential pathophysiological mechanisms have been suggested as to how the pituitary gland is damaged. Location and anatomy of the pituitary gland make it particularly susceptible to injury. The hypophyseal portal vessels and nerve fiber tracks in the infundibular stalk are vulnerable to acceleration and deceleration forces that can result in shearing. The sella turcica is a restricted space that permits pituitary constriction via edema, and

encourages an ischemic environment (Fig. 1).¹⁸ Alterations in pituitary gland morphology have been documented following TBI. MRI shows significant swelling of the pituitary following TBI,^{19,20} whereas postmortem histopathology reveals necrotic lesions within the stalk and pituitary.^{2,21–25}

TBI-induced pituitary injury has been well described, but the adolescent brain may be at the greatest risk. The greater incidence of TBI/repeat TBI (RTBI) and cerebral developmental changes contribute to greater potential pituitary injury in this population. A growing body of evidence also points toward TBI being a cause of hypopituitarism in the adolescent population.²⁶ This is of particular importance, as the highest risk of TBI occurs within the early adolescent and adolescent age groups.²⁷ Disruption of pituitary function could have important clinical consequences, as the neuroendocrine system is an integral part of development. With >1,700,000 annual cases, and a rate of 298/100,000 of those occurring within the adolescent population, TBI is the leading cause of morbidity and mortality among the pediatric age group.²⁷ Whereas incidence of RTBI is difficult to quantify, it appears that approximately one third of those within the adolescent range have received multiple concussions.²⁸ Few studies have been performed comparing the effects of RTBI on brain development, but it has

¹Department of Neurosurgery, ²The UCLA Brain Injury Research Center, ³The Interdepartmental Program for Neuroscience, and ⁴Department of Molecular and Medical Pharmacology, Semel Institute, Los Angeles, California.

FIG. 1. Location and diagram of the pituitary gland. The pituitary is located in the third ventricle in the sella turcica and is connected to the hypothalamus via the infundibular stalk. Area inside circle shows portal vessels and axons from the hypothalamus enter the pituitary through the infundibular stalk. Forces sustained during traumatic brain injury (TBI) causes ischemia as a result of compression from swelling within the sella turcica and shearing of vessels and axons leading to loss of input from the hypothalamus.

been observed that RTBI results in greater learning and memory deficits in adolescents than in those who only sustained a single TBI.^{29–33} These repeat injuries are likely to contribute to a higher rate of pituitary dysfunction. Despite the large and growing number of TBIs, there are currently no experimental studies addressing the occurrence of pituitary dysfunction in adolescents.

In addition to the greater incidence of TBI, the cerebral changes during adolescence represent a unique window of vulnerability to TBI and pituitary dysfunction. Beyond the physical and pubertal developments observed during that time, it is a period of critical

anatomical and functional changes in the brain. Remodeling of cortical and limbic circuitry result in maturation of reproductive, cognitive, social, and emotional axes necessary for proper function during adulthood.³⁴ Many of the developmental processes that occur during gestation are repeated during adolescence. These include neurogenesis, apoptosis, axonal sprouting, myelination, dendritic arborization, retraction, synaptogenesis, synapse elimination, and pruning and sexual differentiation of the brain.^{35,36} Activity of neuroendocrine axes, including GH, peak during adolescence and influence neuronal plasticity and gene expression, resulting in permanent organizational changes within the brain. This developmental stage regulated by pituitary hormones is characterized by rapid changes, and is vulnerable to any kind of perturbation, whether it be injury or environmental. Although causation between isolated hormone deficiencies during childhood/adolescence and perturbed adult function has been shown in several syndromes and experimental models,^{35,37–40} it is yet to be determined how pituitary dysfunction caused by TBI may affect developmental, behavioral, and functional outcome. Currently, no studies address the role of pituitary function in acute or long-term settings following TBI or RTBI in adolescents.

This study tested the hypothesis that exposure to multiple mild TBI results in cumulative damage to the pituitary compared with a single mild TBI that results in disruption of the GH/insulin-like growth hormone 1 (IGF-1) axis. To investigate this hypothesis, the amount of circulating GH or IGF-1 following either a single TBI or four RTBIs was quantified in an adolescent rat. Vascular permeability of the pituitary gland was quantified via Evans Blue dye extravasation. Changes in weight and length of animals were measured as a potential consequence of GH and IGF-1 disruption.

Methods

Subjects

Postnatal day (PND) 35 rats, which age best represents the adolescent development stage, were used in this model. The vast majorities of injuries sustained by adolescents are mild, show no overt pathology, and are a closed head injury. A previously validated closed head RTBI model that does not result in skull fracture or cell death, but does produce the white matter damage and memory deficits commonly seen following mild TBI, was used.⁴¹ PND35 male Sprague–Dawley rats were randomly divided into groups and given sham, single TBI, or RTBI. RTBI animals received four injuries (delivered at 24 h intervals). Adolescent children are at most risk for incurring both mild TBI and RTBI. Although there are no exact parallels of age between species, PND35 rats best represent an adolescent age group based on developmental profiles. Adolescence is a stage that immediately precedes and continues after the onset of puberty.^{36,42} Hallmarks of adolescence include learning and maturation of physical, psychological, social, and cognitive adult behaviors that are dependent upon brain development and neuroendocrine systems.^{34,35,43} In the rat, the adolescent stage is thought to occur between PND 28 and PND 42.⁴² Similarly to human adolescents, during this period rats undergo puberty, progressive and regressive changes in brain volume, maturation of neurotransmitter systems, and increasing production of hormones.^{44–50} Similarly, changes in behavior such as increases in social behavior, risk taking, eating, and sleeping are also observed.^{51–54} All procedures were approved with the UCLA Chancellor's Committee for Animal Research.

Physical characteristics

The weight of animals was monitored, as GH and IGF-1 are responsible for somatic growth. Animals were weighed ($n = 12$) and

TABLE 1. RODENT WEIGHT

Groups	Weight (g)				
	DOI	1 week	2 weeks	3 weeks	1 month
Sham	151±4.63	214±5.81	276±4.61	342±6.98	384±8.96
1TBI	147±5.01	213±7.03	276±8.22	329±9.07	383±9.90
RTBI	142±4.00	209±4.61	253±4.95*	313±7.54*	351±5.10*

Average ($\pm$ SEM) changes of body weight at baseline and after injury.

* $p < 0.05$

DOI, day of injury; TBI, traumatic brain injury; RTBI, repeat traumatic brain injury.

measured ($n=6$) from the tip of the snout to the base of tail. For pituitary gland wet weight, animals ($n=6$) were anesthetized with isoflurane (2.0%/100% O_2) followed by decapitation. Pituitary glands were dissected from the base of skull and immediately weighed.

Closed head injury model

Under isoflurane (2.0%/100% O_2), the animal's head was shaved and placed against a wooden block within a stereotaxic frame without earbars. An electronically controlled pneumatic piston cylinder was mounted onto a stereotaxic micromanipulator to allow for precise localization of the impact center. A mask was used to mark the center of impact (-3AP, -4ML relative to bregma) and the injury tip was firmly zeroed against the skin. The piston was angled at 23 degrees away from the vertical to allow the impactor to make contact perpendicular to the brain surface. The impactor tip (5 mm diameter) displaced the head 8 mm at 36 psi. The head was free to move in the direction of the injury. Injured animals show increases in apnea, and delayed response to toe pinch and righting.⁴¹

IGF-1 and GH quantification

Blood was collected via tail vein into a collection tube containing ethylenediaminetetraacetic acid (EDTA). Samples were spun at 3600 rpm for 20 min within 30 min of collection, and blood plasma was obtained. Blood plasma was then used in an IGF-1 enzyme-linked immunosorbent assay (ELISA) kit, R&D Systems, or in a GH ELISA kit, Invitrogen, according to manufacturer's instructions to quantify circulating levels of both IGF-1 ($n=6$) and GH ($n=10$).

Quantification of Evans Blue dye in the pituitary gland

Animals ($n=6$) were injected intraperitoneally with 7 mL/kg, 2% Evans Blue dye 24 h after injury, which was allowed to circulate for 1 h. Animals were anesthetized with pentobarbital and transcardially perfused with 0.9% saline for 3 min, until the perfusate ran clear. The pituitary was carefully dissected from the base

of the skull and homogenized in 50 μ L of 50% trichloroacetic acid. Samples were kept at room temperature for 5 min and then centrifuged for 15 min at 15,000g at 4°C. The supernatant was removed and diluted 5 $\times$ in 100% ethanol. Evans Blue dye was measured by spectrophotometer at 610 nm and quantified according to a standard curve. The results are presented as ng of Evans Blue dye/mg of tissue.

Statistical analysis

Measurements for weight, length, IGF-1, GH, and Evans Blue dye were analyzed using one way ANOVA for group (sham, single, and RTBI). Post-hoc analyses were conducted with Tukey's post-hoc analysis.

Results

Physical development after TBI

Tables 1 and 2 show changes in weight gain and growth in injury groups over time. There were no significant differences in starting weights or lengths among groups. At 2 weeks following injury, weight gain in RTBI animals was significantly decreased compared with both sham ($p=0.029$) and single TBI ($p=0.033$) groups, and RTBI animals were significant shorter in length than shams ($p=0.027$). RTBI weight and length was still significantly reduced compared with sham at 3 weeks ($p=0.035$ and $p=0.038$, respectively). One month following injury, RTBI animals weighed significantly less than sham ($p=0.023$) and single injury ($p=0.028$) animals; however, length of animals only showed a trend ($p=0.055$) toward being smaller. Single injury alone had no significant effect on weight gain or growth over all time points.

Reduction of circulating GH and IGF-1

There were no significant differences in baseline concentrations of plasma GH among groups. Neither single injury nor RTBI appeared to have affected acute concentrations of GH. At 1 month post-injury, GH was significantly reduced in RTBI animals

TABLE 2. RODENT LENGTH

Groups	Length (cm)				
	DOI	1 week	2 weeks	3 weeks	1 month
Sham	17.7±0.33	20.2±0.26	22.0±0.13	23.6±0.23	24.5±0.31
1TBI	17.8±0.33	20.1±0.15	21.9±0.20	23.3±0.27	24.2±0.22
RTBI	18.2±0.29	20.1±0.27	21.1±0.30*	22.7±0.16*	23.6±0.22

Average ($\pm$ SEM) changes of body length at baseline and after injury.

* $p < 0.05$.

DOI, day of injury; TBI, traumatic brain injury; RTBI, repeat traumatic brain injury.

FIG. 2. Changes in the growth hormone/insulin-like growth hormone 1 (GH/IGF-1) axis after injury. **(A)** Average ($\pm$ SEM) changes in circulating GH at baseline and at 24 h, 72 h, 1 week, and 1 month after single or repeat injury. $*p < 0.05$ **(B)** Average ($\pm$ SEM) changes in circulating IGF-1 at baseline and at 24 h, 72 h, 1 week, and 1 month after single or repeat injury. $*p < 0.05$

compared with sham (Fig. 2A). Baseline concentrations of IGF-1 showed no significant difference among groups. IGF-1 was significantly reduced acutely compared with both sham and single injury at 1 week, and significantly reduced at 1 month, compared with sham (Fig. 2B).

Pituitary wet weight

Pituitary volume increases during adolescence, as production of hormones increases. Pituitary weight was significantly decreased in RTBI animals at 72 h after injury compared with sham and single injury animals (Table 3). Pituitary volume remained smaller, but not significantly, in the RTBI group at 1 week, and there was no difference at 1 month post-injury.

Quantification of Evans Blue dye in the pituitary

Twenty-four hours after sham, single, or repeat injuries, animals were injected with Evans Blue dye to determine vascular disruption within the pituitary. RTBI animals had a significant increase in Evans Blue dye extravasation compared with sham (160% increase) and single injury (130% increase) animals (Fig. 3).

Discussion

This is the first study to show pituitary dysfunction in an adolescent rodent model of repeat mild TBI. Only two prior studies have shown pituitary dysfunction following severe TBI in neonatal or adult rats.^{55,56} The primary conclusion reached is that multiple mild injuries have a cumulative affect that results in disruption of

the GH/IGF-1 axis. This agrees with a clinical case study in an adolescent male who had sustained multiple concussions.⁵⁷ Numerous injury mechanisms that can cause pituitary dysfunction exist, but novel results showing increased Evans Blue dye extravasation suggest a vascular event.

Incidence and time course of hormonal changes

Little is known about pediatric pituitary dysfunction following TBI compared to adults. In the literature there are few studies that observe development of pituitary dysfunction and none address long-term consequences. In the prospective and retrospective studies available, the pediatric population shows an incidence rate of pituitary dysfunction in 16–61% of patients, 1–5 years after injury, compared with the 23–69% seen in adults.^{26,58} Variation in reported rates may be the result of differences in inclusion criteria and diagnostic methods (e.g., baseline testing vs. provocative testing). Nonetheless, pituitary dysfunction following TBI is an important syndrome to identify and treat.

FIG. 3. Repeat traumatic brain injury (RTBI) induced increase in Evans Blue dye extravasation in the pituitary gland. Graph shows the average ($\pm$ SEM) change in Evans Blue dye permeability in the pituitary gland 24 h after sham, single, or repeat injury. $*p < 0.05$

TABLE 3. PITUITARY WET WEIGHT

Groups	Weight (mg)			
	24 h	72 h	1 week	1 month
Sham	7.17 $\pm$ 1.14	7.00 $\pm$ 0.68	9.67 $\pm$ 0.84	12.3 $\pm$ 0.42
1TBI	7.17 $\pm$ 0.95	8.50 $\pm$ 0.76	9.50 $\pm$ 1.45	11.8 $\pm$ 0.31
RTBI	6.83 $\pm$ 0.75	4.50 $\pm$ 0.34*	7.67 $\pm$ 0.49	11.8 $\pm$ 0.83

Average ($\pm$ SEM) changes of pituitary wet weight after injury.

$*p < 0.05$.

TBI, traumatic brain injury; RTBI, repeat traumatic brain injury.

Pituitary dysfunction following TBI has been shown to be both transient and chronic. In children, most cases appear to be diagnosed by 6 months after injury, with the majority of cases resolving within 1 year.^{59–66} In the current experiments, significant decreases in either GH or IGF-1 were not present in single injury animals. In the RTBI group, IGF-1 was significantly reduced at 1 week, and continued to be low at 1 month, whereas GH was only significantly decreased at 1 month after injury. GH ordinarily regulates circulating levels of IGF-1 (i.e., if circulating IGF-1 is low, circulating GH should also be low), however, peripheral GH resistance, shown by normal to elevated levels of GH compared with low levels of IGF-1, has been observed acutely following TBI.¹⁸ Another explanation may be that although baseline levels of GH may not change significantly after injury, this does not account for any alterations in GH pulse amplitude and frequency. The pulse amplitude and frequency are responsible for specific downstream effects of GH, and changes can cause an uncoupling of the GH/IGF-1 system.^{67–69} Alternatively, low IGF-1 in the presence of equivalent levels of GH compared with sham may be an age-dependent phenomenon. IGF-1 production begins to peak independently and prior to GH during adolescence.⁶⁹ As seen in Figure 2, we may be seeing an inhibition in the rise of IGF-1, which would normally happen during this developmental phase.

Consequences of pituitary injury in adolescence

Over the past several years, evidence has shown that mild TBI can result in significant consequences for patients. It results in white matter changes and damage,⁷⁰ and has also been shown to affect cognitive, effective, and somatic abilities of patients.⁷¹ This is of particular relevance, as the risk of TBI increases drastically during adolescence and young adulthood.^{27,28} Within this high-risk group is a subset of patients receiving multiple mild TBIs, children ages 10–14 and adolescents ages 15–19 are two and three times, respectively, more likely to receive a subsequent TBI.⁷² The short- and long-term consequences of RTBI are just beginning to be understood, but it has already been observed that RTBI has cumulative effects, including decreased information processing, increased learning disabilities, and increased difficulty with memory and concentration.^{29–33} An attestation to the vulnerability of the adolescent age group, and the magnitude and duration of symptoms following TBI, are inversely related to age, such that high school athletes have longer memory deficits than do college athletes.^{73,74}

Disruption of the GH/IGF-1 axis during adolescence can have a host of manifestations, as it is involved in several physiological processes including onset of puberty, growth, metabolism, sleep, brain maturation, brain gene expression regulation, social behavior, reproductive capability, and cognitive abilities including memory and executive functioning.^{6,39,69,75–78} Many of the symptoms reported after TBI including fatigue, poor memory and concentration, and irritability are often associated with post-concussive symptoms, and are not typically associated with pituitary dysfunction, although it may be a contributing cause. A further complication may be that many of the post-concussive symptoms reported are changes in behavior that developmentally normal adolescents would display.^{36,79–81} Therefore, a question yet to be answered is how to differentiate between a healthy and an endocrine-deficient patient following TBI. Several pediatric clinical studies have suggested the use of growth and body mass index as independent measures of endocrine dysfunction, to perform dynamic hormone testing.⁸² It would be expected that GH-deficient children would present with stunted growth. In accord to this reasoning, both length

and weight were measured in animals at baseline and then weekly for 4 weeks after injury. At weeks 2–4 after injury, RTBI animals were significantly smaller than sham animals. Although these results were not surprising, given the GH and IGF-1 deficiencies shown in Figure 2, the issue with adopting this as a diagnostic approach is that not all children following TBI with GH deficiencies are of short stature, and not all children of short stature are GH deficient, as per the limited number of studies available.⁸³ Whereas these approaches alleviate concerns of unnecessary and excessive medical testing, they do not address consequences of potentially waiting too long to easily correct hormonal imbalances before leading to potential long-term consequences.

Potential mechanisms of damage and recovery of the pituitary gland

In both pediatric and adult patients, pituitary dysfunction has been observed acutely, and can develop over time.^{26,58} Damage to the pituitary from both primary and secondary injuries is likely to be responsible for the acute impairment and the development of persistent dysfunction seen in many patients. Acute MRI scans and numerous pathological reports have shown evidence of hemorrhage, swelling, infundibular stalk transection, and necrotic lesions in the acute time frames after injury (<1 week).^{19–22} Based on prior publications indicating vascular disruption following TBI, Evans Blue dye permeability was assessed in the pituitary gland 24 h after injury, in order to explain why an injury model that produces no overt brain pathology results in endocrine dysfunction. Evans Blue dye was selected over other histological measures such as hematoxylin and eosin, as it provides a quantitative assessment of injury. A single injury did not result in any changes in permeability; however, RTBI caused a >100% increase in Evans Blue dye permeability indicating “leaky” or damaged vessels within the pituitary. This event could be separated from normal blood–brain barrier breakdown following TBI, as the median eminence and pituitary are some of the few areas of the brain not protected by the blood–brain barrier, and, further, the capillaries within the anterior pituitary are fenestrated, allowing for simple diffusion of hormones.⁸⁴

Although a single impact may not produce enough force to create an injury, RTBI results in acute vascular disruption in the pituitary in the presence of increased inflammatory activity and no cell death.⁴¹ Kasturi and Stein showed persistent inflammation in both the hypothalamus and anterior pituitary gland 2 months after rats received a bilateral frontal controlled cortical impact.⁵⁵ Ongoing inflammatory processes caused by mild repeat injury could be responsible for the development of pituitary dysfunction over time as well as the ongoing dysfunction observed. Further, Tanriverdi et al. have shown development of both anti-hypothalamus and anti-pituitary antibodies in boxers exposed to RTBI, which has been attributed to ongoing pituitary dysfunction.⁸⁵

Significant decrease of the size of pituitary at 72 h following injury could be the result of cell death, and the subsequent increase in size could be indicative of the recovery of cell populations within the pituitary. The pituitary is a homeostatic tissue that can adjust the percentage of cell populations and size in response to stimuli by upregulating production and differentiation of stem cell populations within the gland.⁸⁶ This may be one mechanism by which pituitary function is able to recover over time.

Conclusion

In summary, repeat mild injuries to the head result in both acute and chronic disruption of the GH/IGF-1 axis, whereas results from

the Evans Blue permeability experiment suggest evidence of vascular damage within the pituitary as a cause of dysfunction. It is of urgent need to determine long-term consequences of endocrine disruption during adolescence, as transient disruptions have the potential to alter normal adult behavior.

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Address correspondence to:

Tiffany Greco, PhD

UCLA Department of Neurosurgery

10833 Le Conte Avenue

Semel Institute, Room 18-212

Los Angeles, CA 90095

E-mail: tgreco@mednet.ucla.edu