Novel TRH analog improves motor and cognitive recovery after traumatic brain injury in rodents

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Faden, Alan I., Gerard B. Fox, Lei Fan, Gian Luca Araldi, Lixin Qiao, Shaomeng Wang, and Alan P. Kozikowski. Novel TRH analog improves motor and cognitive recovery after traumatic brain injury in rodents. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R1196–R1204, 1999.—Thyrotropin-releasing hormone (TRH) and certain TRH analogs show substantial neuroprotective effects in experimental brain or spinal cord trauma but also have other physiological actions (autonomic, analeptic, and endocrine) that may be undesirable for the treatment of neurotrauma in humans. We developed a novel TRH analog (2-ARA-53a), with substitutions at the NH2-terminus and imidazole ring, that preserves the neuroprotective action of TRH-like compounds while decreasing or eliminating their autonomic, analeptic, and endocrine effects. Rats administered 2-ARA-53a (1.0 mg/kg, n = 17) intravenously 30 min after lateral fluid percussion brain injury showed marked improvement in motor recovery compared with vehicle-treated controls (n = 14). Treatment of mice subjected to moderate controlled cortical impact brain injury at the same dose and time after trauma (n = 8), improved both motor recovery and cognitive performance in a water maze place learning task compared with vehicle-treated controls (n = 8). In injured rats, no autonomic or analeptic effects were observed with this compound, and endocrine effects were significantly reduced with 2-ARA-53a, in contrast to those found with a typical NH2-terminal-substituted TRH analog (YM-14673). These findings demonstrate that the neuroprotective effects of TRH-related compounds can be dissociated from their other major physiological actions and suggest a potential role for dual-substituted TRH analogs in the treatment of clinical neurotrauma.

N-methyl-D-aspartate antagonists (10), serotonin antagonists (34), and calcium channel blockers (6). TRH has a large therapeutic window: neuroprotective effects have been demonstrated when the compound is administered 24 h or even longer after trauma (11, 13). Moreover, this class of drugs is very well tolerated at even high doses in humans and in a small pilot study showed considerable promise for the treatment of human spinal cord injury (32). There is also evidence that TRH-like compounds may enhance cognitive function (31).

However, TRH and traditional TRH analogs have certain potential limitations as clinical treatment for central nervous system (CNS) trauma. TRH itself has a very short biological half-life of ~5 min in humans, because it is rapidly broken down by endopeptidases (3, 15, 28). TRH also has potent endocrine, analeptic, and autonomic actions (9, 15, 28). The endocrine effects may limit chronic treatment, which may be desirable for its potential nootropic actions. Its autonomic actions include a significant pressor effect (28) that may serve to exacerbate posttraumatic bleeding, an important contributing factor for mortality after traumatic brain injury (TBI). Moreover, under certain conditions, TRH may increase body temperature (28), an undesirable effect in acute brain injury. In addition, its analeptic actions may serve to antagonize the induction of pharmacological coma treatment.

Structure-activity relationship studies suggest that integrity of the COOH-terminus of the tripeptide is critical for the neuroprotective effects of TRH analogs. Thus modification of the COOH-terminus, which yields compounds that show equi-effectiveness with TRH with regard to its other major physiological actions (i.e., endocrine, autonomic, and analeptic), are devoid of neuroprotective effects in CNS trauma, even at doses as high as 10 mg/kg (16). In contrast, modifications at the NH2-terminus yield structures that are neuroprotective yet resist enzymatic degradation by endopeptidases; these compounds have biological half-lives of several hours compared with that of TRH (5 min; see Refs. 16 and 28). However, such analogs also preserve the endocrine, autonomic, and analeptic effects of native TRH (16, 28). Previously, we explored the effects of imidazole substitutions of TRH and found that several such modifications produced compounds that have neuroprotective activity but show less endocrine or autonomic effects than TRH (15).

Collectively, these observations raised the possibility of developing dual-substituted structures (at both the NH2-terminus and the imidazole ring). Theoretically, such compounds should have high CNS activity, resis-
tance to enzymatic degradation (long biological half-life), and, through imidazole modifications, few of the other physiological effects of TRH (i.e., its autonomic, analeptic, and endocrine actions). We have recently tested this concept by synthesizing a dual-substituted TRH analog and evaluating it in two clinically relevant rodent models of TBI. Parallel experiments were performed using lateral fluid-percussion injury in rats and controlled cortical impact (CCI) in mice to determine whether any neuroprotective effects were species or model dependent.

MATERIALS AND METHODS

Chemistry

The structure of the new TRH analog reported in this manuscript, together with that of YM-14673 and TRH, is given in Fig. 1; YM-14673 has been studied extensively in CNS trauma (4, 5, 9, 10, 25, 34). As can be seen from the chemical structures, 2-ARA-53a differs from YM-14673 by the presence of two iodine atoms on the histidine residue. These modifications were intended to alter the lipophilicity of the molecule, thereby improving penetration into the brain. In addition to changing the logP values (see RESULTS), the imidazole substitutions should modify the major physiological actions of TRH (15). Moreover, introduction of the iodine atoms will serve to decrease the ability of the imidazole ring to undergo protonation at a physiological pH. 2-ARA-53a was prepared by direct iodination of YM-14673, purified by high-pressure liquid chromatography, and characterized by a combination of high-field nuclear magnetic resonance analysis, infrared spectroscopy, and electrospray mass spectral analysis.

It has been proposed that the lactam moiety of the pyroglutamyl residue, the histidine imidazole ring, and the carboxamide of the terminal prolinamide act as pharmacophoric groups on the basis of activity in thyroid-stimulating hormone (TSH) release and high-affinity receptor binding (31). To investigate if YM-14673 and the new TRH analog 2-ARA-53a can adopt a low-energy conformation similar to the proposed bioactive conformation of TRH, molecular modeling was performed on these three compounds using the QUANTA/CHARMM modeling package (Molecular Simulations, Waltham, MA). Because the only changes made to 2-ARA-53a relative to YM-14673 are the iodo substitutions on the histidine ring, we have focused our conformational analysis on this portion of the molecules. The two rotatable bonds, which link the histidine ring and the backbone of the molecules, were systematically changed with a step size of 30°. This generated a total of 144 conformations for each molecule. Each of these 144 conformations was then energy-minimized. Cluster analysis on these 144 minimized conformations showed that each molecule has three major conformational clusters. For TRH, two of the three clusters resemble the X-ray conformation, whereas the third major cluster is identical to the proposed active conformation. Superposition of the three conformational clusters identified for YM-14673 and 2-ARA-53a using the pharmacophoric groups showed that one major conformational cluster for both compounds closely resembled the proposed active conformation of TRH, as depicted in Fig. 2. These results suggest that both YM-14673 and 2-ARA-53a can adopt the proposed bioactive conformation of TRH, and the iodo substitutions on 2-ARA-53a do not significantly change the conformational profile of the compound.

Rat Studies

Animals. Male Sprague-Dawley rats (375–425 g) were obtained from Harlan (Frederick, MD) and were housed for at least 1 wk before any procedures. The animals were maintained at a constant temperature (22 ± 2°C) and a 12:12-h light-dark cycle, with lights on at 6:00 AM and all neurological scoring performed during the light cycle. Food and water were available ad libitum.

Fluid-percussion induced TBI. Rats were anesthetized with pentobarbital sodium (70 mg/kg ip) and were intubated and ventilated; femoral venous and arterial catheters were implanted. Brain temperature was assessed indirectly through a thermistor in the temporalis muscle, and body temperature was maintained through a feedback-controlled heating blanket. Blood pressure was continuously monitored, and arterial blood gases were analyzed periodically before trauma. After the animal was placed in a stereotaxic frame, the scalp and temporal muscles were reflected, and a small craniotomy (5 mm) located midway between the lambda and bregma sutures over the left parietal cortex allowed insertion of a Luer-Lok that was cemented in place. The fluid-percussion head injury device, manufactured by the Medical College of Virginia, consists of a Plexiglas cylindrical reservoir filled with isotonic saline; one end includes a transducer that is...

Fig. 1. Structure of 2-ARA-53a, YM-14673, and thyrotropin-releasing hormone (TRH). The presence of 2 iodine atoms on the histidine residue of 2-ARA-53a differentiates this compound from YM-14673.
formed at 1, 7, and 14 days after TBI by individuals unaware of treatment. Motor function was evaluated using three separate tests, each of which is scored via an ordinal scale ranging from zero (severely impaired) to five (normal function). Tests include the ability to maintain position on an inclined plane in the vertical and two horizontal positions for 5 s (forelimb flexion and forced lateral pulsion). Forelimb flexion measures the reflex extension of the forelimb to break a fall when suspended by the tail. Lateral pulsion measures the degree of resistance to a lateral push. Each of seven individual scores (vertical angle, right and left horizontal angle, right and left forelimb flexion, right and left lateral pulsion) were added to yield a composite neurological score ranging from 0 to 35. This scoring method, which has been used in our laboratory extensively during the last 10 yr, shows high inter-rater reliability and is very sensitive to pharmacological manipulations (10).

Analytic and autonomic assessment. Additional groups of uninjured rats were tested for autonomic and analeptic responses immediately before and up to 60 min after drug administration. For the analeptic study, rats were first anesthetized with 40 mg/kg ip pentobarbital sodium and were placed on an unheated pad on the laboratory benchtop at room temperature (22 ± 2°C). A thermistor probe was placed in the rectum to measure core body temperature. After a 10-min period, rats were administered vehicle or drug (each n = 6) as described in Drug administration via the tail vein. Time to recovery of the righting reflex was subsequently determined while temperature was recorded at 5-min intervals for all animals.

To assess autonomic responses to the new TRH analog, a separate group of rats was anesthetized with 4% isoflurane (1.5 l/min). Catheters were then placed in the right carotid artery and right jugular vein and exteriorized at the back of the neck. Rats were separated one per cage and allowed to recover from anesthesia. The exteriorized catheters were suspended above the rat to prevent biting. Mean arterial blood pressure (MAP) was continuously recorded via a transducer connected directly to the arterial catheter for the duration of the study. At 1 h after catheter placement, rats were administered vehicle or drug via the catheter in the jugular vein as described in Drug administration.

Endocrine assessment. An RIA for rat (r) TSH was used to assess endocrine function. Anesthetized rats (70 mg/kg pentobarbital sodium; n = 6/group) were administered either saline, YM-14673, or 2-ARA-53a intravenously via a chronically implanted catheter at time 0, and 5 ml of whole blood were collected 30 min after injection. Blood plasma was subsequently obtained, frozen at −70°C, and shipped to Covance Laboratories (Vienna, VA) for appropriate analyses. The rTSH assay is a specific double-antibody RIA utilizing antiserum prepared in the rabbit against purified rTSH antigens (22). The assay is standardized against purified rTSH.

Drug administration. For TBI studies, rats were injected via the femoral vein catheter with either 1.0 ml/kg of normal saline (n = 14) or 1.0 mg/kg 2-ARA-53a (n = 17) at 30 min after TBI. The investigator was blinded to drug treatment both at the time of surgery and for neurological scoring. For autonomic and anelpteic studies, rats were given either 1.0 ml/kg of normal saline (n = 6), 1.0 mg/kg of YM-14673 (n = 6), or 1.0 mg/kg 2-ARA-53a (n = 6) at the times indicated in Analytic and autonomic assessment.

Mouse Studies

Animals. Male C57BL/6 mice (20–25 g) were obtained from Taconic Farms and were housed in an area directly adjoining surgical and behavioral rooms for at least 1 wk before any

mounted and connected to a 5-mm tube that attaches through a male Luer-Lok fitting to the female Luer-Lok cemented at the time of surgery. A pendulum strikes a piston at the opposite end of the device, producing a pressure pulse of ~22 ms duration, leading to the deformation of underlying brain. The degree of injury is related to the pressure pulse expressed in atmospheres (atm): 2.6 atm in our laboratory produces a moderate injury with regard to neurological and histological deficit (10, 27). Sham (control) animals underwent anesthesia and surgery without fluid percussion brain injury.

Neurological scoring. Standardized motor scoring was performed at 1, 7, and 14 days after TBI by individuals unaware of the low energy conformation of YM-14673 (dark) on the bioactive conformation of TRH (light). These results suggest that both YM-14673 and 2-ARA-53a can adapt the proposed bioactive conformation of TRH and that the iodo substitutions on 2-ARA-53a do not significantly change the conformational profile of the compound.

Fig. 2. A: superposition of the proposed bioactive conformation of TRH (dark) on the X-ray structure of TRH (light). B: superposition of the low energy conformation of YM-14673 (dark) on the bioactive conformation of TRH (light). C: superposition of the low energy conformation of 2-ARA-53a (dark) on the bioactive conformation of TRH (light). These results suggest that both YM-14673 and 2-ARA-53a can adapt the proposed bioactive conformation of TRH and that the iodo substitutions on 2-ARA-53a do not significantly change the conformational profile of the compound.
procedures. All mice were maintained at a constant temperature (22 ± 2°C) and a 12:12-h light-dark cycle, with lights on at 6:00 AM and all behavioral testing performed during the light cycle. Food and water were available ad libitum.

CCI device. Our injury device was designed and built at the Georgetown Institute for Cognitive and Computational Sciences and consists of a microprocessor-controlled pneumatic impactor with a 3.5-mm-diameter tip (19). The impactor was vertically mounted on a mill table (Sherline) that allowed for precise adjustment in the vertical plane above the mouse head, which itself was secured to a stereotaxic apparatus (David Kopf Instruments) attached to the instrument. The core rod of a linear voltage differential transducer (LVDT; Serotec) was attached to the lower end of the impactor to allow measurement of velocities between 3.0 and 9.0 m/s. Velocity of the impactor was controlled by fine tuning both positive and negative (back) air pressures. An oscilloscope (Tektronix) recorded the time/displacement curve produced by the downward force on the LVDT, allowing precise measurement of the impactor velocity.

Surgery. Surgical anesthesia was induced and maintained with 4 and 2% isoflurane, respectively, using a flow rate of 1.0–1.5 l oxygen/min. Depth of anesthesia was assessed by monitoring respiration rate and palpebral and pedal-withdrawal reflexes. The animal was then placed on a heated pad, and core body temperature was monitored and maintained at 38 ± 0.2°C. The head was mounted in a stereotaxic frame, and the surgical site was dipped and prepared with a series of three Nolvasan scrubs followed by sterile saline rinses. A 10-mm midline incision was made over the skull, the skin and fascia were reflected, and a 4-mm craniotomy was made on the central aspect of the left parietal bone with a tissue punch (Roboz). Great care was taken with the removal of the parietal bone to avoid injury to the underlying dura mater, which was continuously bathed in sterile normal saline warmed to 37.5°C. The impounder tip of the pneumatic injury device was cleaned with a pad soaked in absolute alcohol, warmed to 37.5°C, and the impounder tip of the pneumatic injury device was then placed on the exposed dura, and reset to impact the cortical surface at a moderate level (6.0 m/s velocity, 1 mm tissue deformation). After injury at a moderate (6.0 m/s velocity, 1 mm tissue deformation) level, the incision was closed with interrupted 6-0 silk sutures, anesthesia was discontinued, and the mouse was placed into a heated cage to maintain normothermia for 45 min postinjury. All animals were monitored carefully for at least 4 h postsurgery and then daily. To minimize variation between animals due to anesthesia duration, the mouse was placed in a heated cage in the supine position. Acute neurological recovery was assessed immediately after placement on the beam.

Spatial learning and memory evaluation. The Morris water maze was employed to assess spatial learning and working memory by training mice to locate a hidden, submerged platform using extramaze visual information. The apparatus used consists of a large, white circular pool (900 mm diameter, 500 mm high, water temperature 24 ± 1°C) with a Plexiglas platform 76 mm in diameter painted white and submerged 15 mm below the surface of water (225 mm high), which is rendered opaque with the addition of dilute, white, nontoxic paint (19). During training, the platform was hidden in one quadrant 14 cm from the side wall. The mouse was gently placed in the water facing the wall at one of four randomly chosen locations separated by 90°. The latency to find the platform was recorded by a blinded observer. On the first trial, mice failing to find the platform within 90 s were assisted to the platform. Animals were allowed to remain on the platform for 15 s on the first trial and 10 s on all subsequent trials. There was an intertrial interval of 30 min, during which time the mice were towel-dried and placed under a heat lamp. A series of 16 training trials administered in blocks of four were conducted on days 7, 8, 9, and 10 postsurgery. This methodology has been shown by us to readily distinguish brain-injured versus sham-injured animals (19).

Drug administration. Conscious mice were placed in a mouse restrainer and were injected via the lateral tail vein with either 1.0 ml/kg of normal saline (n = 8, sham and n = 8, CCI) or 1.0 mg/kg of 2-ARA-53a (n = 8, CCI) at 30 min after CCI injury. The investigator was blinded to drug treatment groups and monitoring of reflexes. The animal was then placed on a heated pad, and core body temperature was monitored and maintained at 38 ± 0.2°C. The head was mounted in a stereotaxic frame, and the surgical site was dipped and prepared with a series of three Nolvasan scrubs followed by sterile saline rinses. A 10-mm midline incision was made over the skull, the skin and fascia were reflected, and a 4-mm craniotomy was made on the central aspect of the left parietal bone with a tissue punch (Roboz). Great care was taken with the removal of the parietal bone to avoid injury to the underlying dura mater, which was continuously bathed in sterile normal saline warmed to 37.5°C. The impounder tip of the pneumatic injury device was cleaned with a pad soaked in absolute alcohol, warmed to 37.5°C, and the impounder tip of the pneumatic injury device was then placed on the exposed dura, and reset to impact the cortical surface at a moderate level (6.0 m/s velocity, 1 mm tissue deformation). After injury at a moderate (6.0 m/s velocity, 1 mm tissue deformation) level, the incision was closed with interrupted 6-0 silk sutures, anesthesia was discontinued, and the mouse was placed into a heated cage to maintain normothermia for 45 min postinjury. All animals were monitored carefully for at least 4 h postsurgery and then daily. To minimize variation between animals due to anesthesia during acute neurological testing, 20 min were allowed for surgery, and 5 min were allowed for suturing each animal.

Acute and chronic neurological evaluation. After cessation of anesthesia, the mouse was placed in a heated cage in the supine position. Acute neurological recovery was assessed in all mice by recording the time to recovery of hind paw flexion after application of pressure, an indicator of simple somatomotor function. Similarly, latency to recovery of the righting reflex, an indicator of somatosensory function, was recorded for each animal.

Chronic neurological recovery was evaluated for all animals using a beam walking task, a method that is particularly good at discriminating fine motor coordination differences between injured and sham-operated animals (19). The device consists of a narrow wooden beam 6 mm wide and 120 mm in length that is suspended 300 mm above a 60-mm-thick foam rubber pad. The mouse was placed on one end of the beam, and the number of footfaults for the right hindlimb was recorded over 50 steps counted in either direction on the beam. Mice were allowed to walk across the beam two times for training purposes before surgery. A basal level of competence at this task was established, with an acceptance level of <10 faults/50 steps. In all subsequent trials after surgery, performance was assessed immediately after placement on the beam.

RESULTS

Chemistry

It is known that hydrophobicity of a drug molecule may play an important role with regard to its cellular permeability. To investigate the hydrophobicity of 2-ARA-53a, YM-14673, and TRH, their partition coefficients between n-octanol and water (logP) were calculated with the Hint Program (edusoft, Ashland, VA). The logP values for TRH, YM-14673, and 2-ARA-53a are -5.46, -5.37, and -3.37, respectively. These values showed that YM-14673 is slightly more hydrophobic than TRH, whereas 2-ARA-53a is at least two orders of magnitude more hydrophobic than TRH and YM-14673. The superior hydrophobicity of 2-ARA-53a would enhance its cellular permeability thereby improving the accessibility of this compound to the brain.
Rat Studies

Physiological responses. Baseline pH, $P_O_2$, and $P_CO_2$ were within the normal range and did not differ between control and treatment groups at the time of injury; these values were maintained in the normal range after trauma through controlled respiration. Preinjury MAP was also similar in control and treatment groups (Table 1). There was a modest decline in MAP after trauma, which did not differ between control and treatment groups (101 ± 6 vs. 99 ± 6 at 5 min; 97 ± 6 vs. 98 ± 7 at 10 min).

Neurological scoring. Moderate lateral fluid percussion injury induces significant deficits of motor function in untreated rats after injury, with a partial, but incomplete, recovery of function occurring over 2 wk. When compared with saline-treated controls, significant improvements in contraflexion score (Fig. 3A) at 7 days ($P = 0.0064$, Mann-Whitney U-test) and 14 days ($P = 0.0467$) after injury, contrapulsion score (Fig. 3B) at 7 days ($P = 0.0179$) postinjury, and angleboard score (Fig. 3C) 14 days after injury ($P = 0.0274$) were observed in rats treated with 2-ARA-53a. Similarly, composite neuroscores (Fig. 3D) for 2-ARA-53a-treated rats were significantly improved at both 7 days ($P = 0.0061$) and 14 days ($P = 0.0229$) after injury.

Mortality. In this study, 3 out of 14 rats given normal saline died before the end of the study. In comparison, a mortality rate of 4 out of 17 was recorded for animals treated with 2-ARA-53a (not statistically significant from controls; $P > 0.05$, chi square).

Autonomic and analeptic studies. Core body temperature, assessed by rectal thermistor probe from sedated rats on an unheated pad (different from trauma studies in which body temperature was maintained at 37.5°C), was similar for all treatment groups immediately after loss of the righting reflex. However, over the following 60 min, body temperature dropped by almost 3°C for rats treated with normal saline or 2-ARA-53a (Fig. 4).

Table 1. Baseline arterial blood gas and mean arterial pressure data

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<th>Control</th>
<th>1-ARA-53a</th>
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<tr>
<td>pH</td>
<td>7.44 ± 0.01</td>
<td>7.44 ± 0.01</td>
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<tr>
<td>$P_O_2$, mmHg</td>
<td>88 ± 2</td>
<td>89 ± 3</td>
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<tr>
<td>$P_CO_2$, mmHg</td>
<td>42 ± 1</td>
<td>41 ± 1</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>114 ± 2</td>
<td>112 ± 5</td>
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Values are means ± SE. MAP, mean arterial blood pressure; 1-ARA-53a, thyrotropin-releasing hormone analog.
In contrast, animals treated with the positive control YM-14673 maintained a core temperature between 37 and 38°C. A repeated-measures ANOVA yielded a significant group effect [$F(2,15) = 27.011, P < 0.0001$], time effect [$F(12,180) = 168.273, P < 0.0001$], and group x time interaction [$F(24,180) = 48.760, P < 0.0001$]. Post hoc analysis with Tukey's pairwise comparison detected significant differences between YM-14673-treated rats and all other groups at time points 20–60 min after loss of the righting reflex ($P < 0.001$). Similar results were obtained when animals were treated with 10 mg/kg iv of drug (data not shown).

Recovery of the righting reflex did not differ significantly between rats treated with saline or 2-ARA-53a, although latency to reflex recovery was significantly attenuated in rats administered YM-14673 (Fig. 5; $P < 0.0001$, Bonferroni post hoc test). This result demonstrates the analeptic effects of YM-14673 and lack thereof in vehicle-treated and 2-ARA-53a-treated animals. MAP did not differ significantly between rats treated with vehicle or 2-ARA-53a (Fig. 6). However, animals administered YM-14673 maintained a significantly higher MAP over the duration of testing compared with all other groups. A repeated-measures ANOVA yielded a significant group effect [$F(2,16) = 4.927, P = 0.0215$]. Post hoc analysis with Tukey’s pairwise comparison detected significant differences between YM-14673-treated rats and all other groups at 60 and 120 min after injection ($P < 0.05$) and between YM-14673 and 2-ARA-53a at 30, 40, 70, 90, 100, and 110 min after injection ($P < 0.05$).

Endocrine effects. Basal levels of TSH (<2 ng/ml) were recorded for all animals administered normal saline. In contrast, TSH levels were significantly elevated in animals treated with YM-14673 when compared with controls ($P < 0.0001$, ANOVA followed by Bonferroni correction; Fig. 7). Rats treated with 2-ARA-53a showed a significantly reduced TSH level when compared with the YM-14673-treated animals ($P < 0.0001$), although levels remained elevated when compared with vehicle controls ($P < 0.0001$).

**Mouse Studies**

Acute recovery. After cessation of anesthesia, sham-operated control mice quickly recovered the pedal withdrawal reflex, whereas untreated animals receiving a moderate injury recovered this reflex more slowly (14.75 ± 1.22 vs. 27.75 ± 1.53 s, $P < 0.0001$, Bonferroni post hoc test). Injured mice treated with 2-ARA-53a did not differ significantly from their saline-treated injured counterparts (26.63 ± 2.20, $P = 0.05$). Recovery of the righting reflex was significantly delayed for saline-treated injured animals when compared with sham-operated controls (176.00 ± 9.33 vs. 66.63 ± 5.65 s, $P < 0.0001$, Bonferroni post hoc test). Injured mice subse-
quently treated with 2-ARA-53a did not differ significantly from saline-treated injured mice (168.38 ± 11.5, P > 0.05).

Beam walking. The number of contralateral rear footfaults was markedly increased in injured animals when compared with sham-operated controls, reaching a maximum 1 day after injury (Fig. 8). Mice treated with 2-ARA-53a began to show a significant recovery of function after 7–14 days and were performing considerably better on this task 3 wk after injury. This is in contrast to saline-treated injured animals, which exhibited significant deficits for the duration of the experiment. The sham-operated group maintained a baseline number of footfaults over the entire testing period (Fig. 8). A repeated-measures ANOVA yielded a significant group effect [F (2,21) = 106.347, P < 0.0001], day effect [F(6,120) = 65.614, P < 0.0001], and group × day interaction [F(12,120) = 17.560, P < 0.0001]. Post hoc analysis with Tukey’s pairwise comparisons detected significant differences between sham-operated and saline-treated injured animals at days 1, 2, 3, 7, 14, and 21 (P < 0.001). However, a significant improvement in performance of this task was observed in mice treated with 2-ARA-53a at 14 (P < 0.05) and 21 (P < 0.01) days after injury when compared with saline-treated injured animals.

Place learning. The effect of CCI on place learning in the water maze task was assessed by comparing the daily mean latency (+ SE) with goal location over the four trials for each group. A clear difference in learning/memory ability emerged between sham-operated and saline-treated injured animals when trained at days 7–10 postsurgery, with sham animals locating the hidden goal platform on a consistently faster basis than their injured counterparts (Fig. 9). A repeated-measures ANOVA yielded a significant group effect [F(2,21) = 27.442, P < 0.0001], day effect [F(6,120) = 29.914, P < 0.0001], and group × day interaction [F(6,120) = 4.681, P = 0.0005], averaged over the 4 days. Post hoc analysis using Tukey’s pairwise comparison detected significant differences between sham animals and saline-treated injured controls on day 8 (P < 0.001), day 9 (P < 0.01), and day 10 (P < 0.001) after injury. On day 8, injured animals given 2-ARA-53a were also significantly different from sham-operated controls (P < 0.001). However, after the third day of training (day 9), 2-ARA-53a-treated animals were outperforming their saline-treated injured counterparts and were no longer significantly different from sham-operated controls (P > 0.05). On the last day of training, a significant improvement in performance, demonstrated by decreased latencies to find the hidden goal platform, were observed for mice treated with 2-ARA-53a (P < 0.01) when compared with saline-treated injured mice.

**DISCUSSION**

Our findings indicate that a TRH analog, with substitutions at both the NH2-terminus and imidazole ring, significantly improves motor and cognitive outcome after experimental TBI. The fact that significant beneficial effects were found with 2-ARA-53a treatment in both the rat fluid percussion model and the mouse CCI model indicates that the protective effects are neither species nor model specific. Fluid-percussion injury and CCI have been developed to reflect different components of clinical head injury and show different pathological and histological features (33). The rat lateral fluid percussion model was developed by us as an alternative to various models in cats (27) and has been widely used to study both pharmacology and pathobiology. Our CCI model has been well characterized in terms of both behavior and histopathology (19) and shares many features of other rodent CCI models.

2-ARA-53a has a substituted imidazole structure identical to that of 2,4-diiodo-TRH, which we have previously shown to improve outcome in the same rat TBI model (15); here we show that this substitution markedly alters the hydrophobicity of the compound. At its NH2-terminus, 2-ARA-53a has a substitution ([S]-2-azetidinone-4-carbonyl) similar to that of YM-14673. Dose-response studies were not conducted, but rather a single intravenous dose of 1 mg/kg was chosen.
Previous structure-activity studies, using a variety of TRH analogs, have shown similar dose-response curves with regard to neuroprotection (a shallow inverted U-shaped dose-response pattern between 0.1 and 10 mg/kg with optimal effects at a dose of 1 mg/kg; see Refs. 9 and 16).

Findings in our mouse CCI injury model demonstrate that animals treated with 2-ARA-53a perform significantly better than saline-treated controls in a beam walking task that assesses fine motor coordination. This improvement was particularly evident 2–3 wk after injury, the last time points evaluated in this study. Cognitive performance in a standard water maze spatial learning task was also significantly improved in these same animals when compared with sham-operated controls 7–10 days after surgery. Although the ability of TRH to enhance cognitive function has been previously reported, the mechanisms involved remain speculative (31). However, possible nootropic effects of 2-ARA-53a would not account for improved performance in our studies, as each animal received only a single dose of the TRH analog (1.0 mg/kg iv) at 30 min after injury. Using histochemical techniques, we have previously shown that moderate CCI injury in the same mouse strain produces both primary and secondary injury to the somatosensory and motor cortex and secondary injury to the hippocampus, causing deficits in beam walking and water maze tasks in an injury-dependent manner (19). In the present study, therefore, improved performance in motor and cognitive tasks likely results from the compound’s neuroprotective effects on cells in these regions. Quantitative histological assessment was not performed but will be required to substantiate specific neuroprotective effects in these TBI models. However, preliminary studies using a well-established in vitro model of traumatic neuronal injury (28, 30) show that dual-substituted TRH-related compounds have significant neuroprotective properties and appear to attenuate both necrotic and apoptotic pathways (unpublished observation).

Although we have not addressed the mechanisms involved in 2-ARA-53a-related neuroprotection, TRH and TRH analogs modify multiple components of the delayed injury cascade that leads to secondary posttraumatic neuronal cell loss (11). This includes the ability to antagonize at a physiological level the actions of endogenous opioids (21), platelet-activating factor (18), leukotrienes (23), and excitatory amino acids (35). These compounds also have effects on CNS blood flow, cellular bioenergetic state, and ionic homeostasis (17, 26, 37). In preliminary studies, a more recently developed dual-substituted TRH analog protected neurons in rat neuronal/glial cell cultures from glutamate and maitoxin-induced neurotoxicity and from staurosporine-induced apoptosis (unpublished data). Thus the beneficial effects of TRH-like compounds likely reflect multifactorial actions.

In contradistinction to TRH and most TRH analogs, 2-ARA-53a has no effects on blood pressure, heart rate, body temperature, or pentobarbital sodium narcosis. Moreover, although it increases TSH activity, the endocrine effects were significantly less than for an NH₂-terminal-substituted TRH analog like YM-14673. These physiological effects (autonomic, analeptic, and endocrine), normally found with TRH, contribute to the major side effects of this class of drugs. Therefore, the absence of such actions suggest that our new analog would be well tolerated, even with chronic use as may be required for treatment of cognitive dysfunction. The lack of a pressor effects would make this compound safer than TRH or traditional TRH analogs for use in severe head injury, where associated bleeding is common. The absence of an eptic actions would allow such compounds to be used potentially even where there is consideration of pharmacological coma therapy. The lack of a thermoregulatory action may also permit concurrent use of hypothermic therapy, if desired. Moreover, with more recently developed dual-substituted analogs, we have been able to entirely eliminate endocrine effects. The present findings clearly demonstrate that the neuroprotective effects of TRH-related compounds may be dissociated from their other major physiological actions. This is consistent with the observation that the neuroprotective effects of TRH analogs are not correlated with activity at high-affinity TRH receptors (unpublished data).

In summary, we have developed a new TRH analog with substitutions at both the NH₂-terminus and imidazole ring that significantly improves motor and cognitive outcome after TBI, yet has no autonomic or analeptic effects and reduced endocrine actions. Compounds of this type should be better tolerated than TRH or traditional TRH analogs and may have a role in the treatment of clinical neurotrauma.

TRH and certain TRH analogs show striking neuroprotective effects in many experimental models of CNS trauma. However, other physiological effects of TRH/TRH analogs, particularly their autonomic and analeptic actions, may serve to limit the potential benefits of treatment. We have developed a TRH analog (2-ARA-53a) with substitutions at both the NH₂-terminus and histidine residue of the tripeptide. The imidazole substitutions markedly increase the hydrophobicity of the compound while eliminating its autonomic and analeptic properties. A single injection of 2-ARA-53a after injury significantly improved behavioral recovery in rats subjected to fluid percussion-induced TBI and mice subjected to CCI brain injury. Although the mechanism(s) of the neuroprotective action of this compound were not examined in the present experiments, recent preliminary studies from our laboratory have shown that dual-substituted tripeptide and dipeptide derivatives related to TRH can reduce both necrotic and apoptotic neuronal cell death in mixed neuronal/glial cultures from rat cortex. Future studies are needed to more precisely delineate neuroprotective mechanisms and the therapeutic window for these compounds.

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This study was supported in part by Center for Disease Control Grant CRC 306634 and DOD DAMD 17–93-V-3018. A. I. Faden is the inventor of an existing patent that includes 2-ARA-53a; this patent was assigned to Georgetown University. A. I. Faden and A. P. Kozikowski have received research support and have consulted for a company (TRH Development Company) that is negotiating with Georgetown University for license rights to various new TRH analogs, including 2-ARA-53a. Requests for reprint requests and other correspondence: A. I. Faden, Georgetown Institute for Cognitive and Computational Sciences, Georgetown Univ Medical Center, 3970 Reservoir Road, NW, Rm. EP-04, Washington, DC 20007-2197 (E-mail: fadena@giccs.georgetown.edu).

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