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Antidepressant effects of thyrotropin-releasing hormone analogues using a rodent model of depression

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This paper is dedicated to T. George Bidder, MD, late of Wales and of this earth, who was and is an exemplar of humanism and scholarship in the practice of medicine. *Holl amrantau'r ser ddywedant Ar hyd y nos*

Abstract

The antidepressant potential of two naturally occurring analogues of thyrotropin-releasing hormone (TRH), pGLU-GLU-PRO-NH_2 (EEP) and pGLU-PHE-PRO-NH_2 (EFP), were examined using a rodent model of antidepressant efficacy. The Porsolt Swim Test was used to assay the antidepressant properties of these two peptides. Both analogues of TRH produced significant antidepressant effects, with EEP producing the stronger response. No effect of EEP upon triiodothyronine (T_3) was observed at the dosage used. EFP, which has previously been demonstrated to crossreact with the TRH receptor, significantly increased serum T_3. Since an effect upon T_3 was only observed in the weaker of the two compounds, these data suggest that the behavioral effect of EEP was not secondary to stimulation of thyroid hormone. Additionally, the differential behavioral response to the two compounds suggests a degree of sequence specificity in the ability of TRH-like tripeptides to produce an antidepressant effect.

Keywords: Depression; TRH; ECS; Porsolt test; Antidepressant treatment

1. Introduction

Recent evidence suggests that thyrotropin-releasing hormone (pGLU-HIS-PRO-NH_2, TRH) may play a role in the development and treatment of depression. TRH has been found to be elevated in the cerebrospinal fluid of patients suffering from major depression (Banki et al., 1988). While, initially, this elevation may suggest a causal influence of TRH, it has also been observed that intravenous administration of TRH produces rapid, but transient, relief from depression (Kastin et al., 1972; Prange et al., 1972), and that intrathecal administration of TRH can produce relief for 1–2 days (Callahan et al., 1997; Marangell et al., 1994, 1997). Hence, the elevated levels of TRH observed in depressed individuals may represent a compensatory response to the illness. In addition to its potential as an endogenous antidepressant, TRH has also been observed to have anticonvulsant properties (Kubek et al., 1989). Consistent with this finding, carbamazepine has been reported to alter levels of TRH mRNA and TRH receptor binding in limbic structures (Rosen et al., 1994).

In patients who suffer from major depression, and who are refractory to a variety of antidepressant pharmacologies, and combinations thereof, fully 50% demonstrate a clinical response (Devan et al., 1991; Prudic et al., 1990) to electroconvulsive therapy (ECT). In addition to its antidepressant efficacy, another striking effect of ECT is its ability to raise seizure threshold; across a course of several treatments, current strength must be progressively augmented in order to produce a seizure (Prudic et al., 1990; Sackeim et al., 1983). In rodent preparations, electroconvulsive shock (ECS) retards the development of kindled seizures, and repeated administrations of ECS have been found to result

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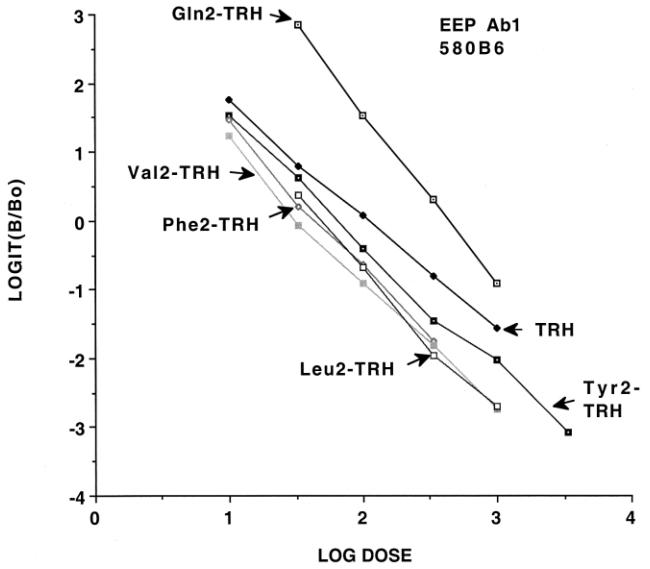

Fig. 1. Analysis of the displacement of ^{125}I-TRH from binding to EEP antiserum 580B4 by serially diluted TRH and TRH-like peptides using a parallel line and relative potency program (Pekary, 1979). Logit(B/B_o) = ln[$(B/B_o)/(1 - B/B_o)$] where B/B_o is the ratio of the counts bound at a finite dose of unlabeled peptide divided by the counts bound at zero dose of unlabeled peptide. The units of the x axis refer to both the concentration of the assay standard (EEP) and the concentrations of the other related peptides.

in progressively shorter clonus duration (Lloyd and Sattin, submitted; Sackeim et al., 1991). Indeed, some have argued that an increase in seizure threshold is necessary for ECT to have an antidepressant effect (Sackeim, 1995; Sackeim et al., 1986, 1987, 1991) although a dissociation between the antidepressant, antiepileptogenic, and anticonvulsive properties of ECS has been demonstrated (Lloyd and Sattin, submitted; Sattin and Lloyd 1993).

It has been found that TRH is synthesized in limbic areas of the brain (Kubek and Sattin, 1984; Kubek et al., 1993; Sattin et al., 1987), suggesting a role as a neuromodulator, independent of its participation in the hypothalamic regulation of thyroid function. When ECS is given to rats, an increase in TRH, and its immediate tetrapeptide precursor (TRH-Gly), has been observed in the limbic, but not the motor, areas of the brain (Sattin and Pekary, 1992; Sattin et al., 1987).

The Porsolt Swim Test (Porsolt et al., 1977, 1978) is an established instrument used by pharmaceutical companies to screen new compounds for potential antidepressant properties. Rats are first placed in an inescapable tank of water for 15 min. The next day, the proportion of time in which they are immobile, across a 5-min interval, is assessed in the same water tank. When treatments are given in a semichronic manner, all clinically effective antidepressant compounds reduce the duration of immobility in this assay. A similar reduction in immobility is found when a series of ECSs are administered.

In the preparations cited above, where ECS produced regionally specific increases in TRH-related peptides, ECS was found to have an antidepressant effect, as indexed by a decrease in immobility in the Porsolt Swim Test. In addition, the animals' swim scores, the index of antidepressant effect, were significantly correlated with limbic levels of TRH and TRH-Gly, while no such correlations were found in other brain areas (Pekary et al., 1994, 1997; Sattin and Pekary, 1992; Sattin et al., 1994). Finally, the TRH-enhancing peptide PS4 (Prepro-TRH[160–169]) has also been found to be selectively elevated in limbic regions by ECS, and the levels of this peptide are also correlated with the antidepressant effect of ECS, as measured by the Porsolt Test (Pekary et al., 1997). These data suggest that the effect of ECS/ECT is to stimulate production of the parent peptide (prepro-TRH), which contains the PS4 sequence and multiple copies of the TRH-Gly sequence (Lechan et al., 1986). Since ECS/ECT and TRH share antidepressant and antiictal properties, and since ECS stimulates the production of TRH, it is possible that the effects observed with ECS/ECT are mediated through its induction of TRH.

A naturally occurring tripeptide, pGLU-GLU-PRO-NH_2 (EEP), has been identified, which is an analogue of TRH. This peptide is not derived from prepro-TRH, and it is not subject to breakdown by TRH metabolizing enzymes in serum (Lechan et al., 1986; Cockle et al., 1994). Systemic application of this peptide suggests that it crosses the blood–brain barrier and that it has dopaminergic properties (Mabrouk and Bennett, 1997). A recent study has further characterized the brain uptake of EEP and has demonstrated that ECS induces the production of EEP in a variety of limbic areas, while other brain regions are unaffected (Pekary et al., 1999). Thus, the antidepressant effects of ECS/ECT may be due, in part, to the induction of EEP, as well as TRH. This peptide is not subject to rapid breakdown by TRH metabolizing enzymes (Cockle et al., 1994). If EEP proves to have antidepressant properties, then this biological substance would have the potential to be a practical antidepressant agent. The present study investigates the antidepressant potential of EEP and another naturally occurring tripeptide, (pGLU-PHE-PRO-NH_2, EFP), which is also related to TRH, using the Porsolt Swim Test. To address the possibility that the behavioral effects of these compounds are secondary to stimulation of

Table 1
Relative potencies for displacement of assay tracer (^{125}I-TRH) by TRH and other TRH-like peptides when measured relative to the EEP assay standard (relative potency = 1.00) as determined in the EEP RIA with the aid of a previously described computer program (Pekary, 1979). Ab_1: antibody

Ab1	Tracer	TRH	Gln2-TRH	Try2-TRH	Phe2-TRH	Val2-TRH	Leu2-TRH	EEP
580B4	^{125}I-TRH	2.91	1.00	5.06	6.53	9.24	5.00	1.00

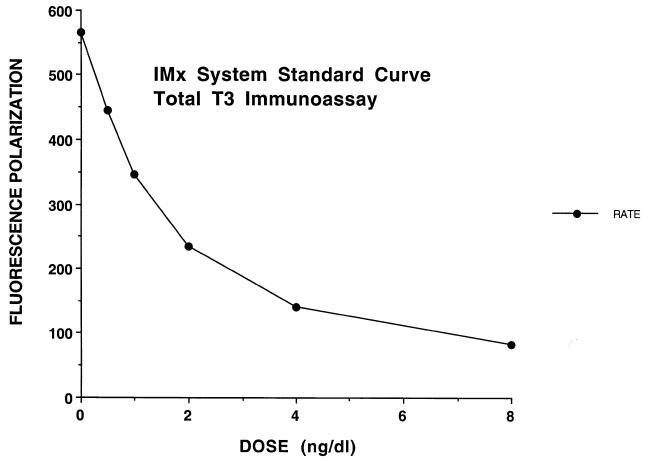

Fig. 2. This fully automated, nonradioisotopic procedure calculates T_3 concentration by measuring the fluorescence polarization of a fixed concentration of fluorescent analog of T_3, which is mixed with T_3 standards or serum samples. In the absence of unlabeled T_3, most of the labeled T_3 is bound to T_3 antibody that has a very slow rotational diffusion rate. With increasing unlabeled T3 concentration in standards or serum samples, labeled T_3 is displaced from the antibody resulting in higher rotational diffusion rate and reduced fluorescence polarization (Pekary and Hershman, 2001).

thyroid function, serum triiodothyronine (T_3) was measured in these animals.

2. Method

2.1. Subjects

Male Wistar rats (Harlan Sprague Dawley, Indianapolis, IN) weighing 180–200 g were housed two to a cage and maintained on a 12-h/12-h light/dark schedule in an AALAC-approved facility. Food and water were provided ad libitum.

2.2. Experimental procedures

On the first day, each animal was individually placed in a clear, inescapable, Plexiglas tank of water for 15 min (preswim). The tank was 25 cm in diameter and 45 cm in height and filled to a depth of 30 cm (25 ± 1°C). Following exposure to the tank, the animal was hand-dried with a towel and injected with either the experimental compound or a matched volume of saline. The animals were run in pairs, with one animal from each cage receiving the experimental compound, and the cage mate receiving saline. Treatment selection was made at random. Each animal received similar injections 5 h following the preswim and 24 h after the preswim. One hour following the last of the three injections, each animal was individually placed in the water tank for 5 min (swim test). During this procedure, the amount of time that the animal spent swimming was recorded using a stopwatch. The immobility score was the difference between this time and the 5-min swim test interval. The only difference between the preswim and swim test procedures, other than the duration, was that the tank was filled to a depth of 15 cm during the swim test.

Following the swim test, the animal was dried with a towel, decapitated, and trunk blood collected. The blood was subsequently centrifuged and the supernatant frozen at − 70°C. Immediately upon decapitation, five brain regions (anterior cortex, pyriform cortex, amygdala/entorhinal cortex, hippocampus, and striatum) were rapidly hand dissected

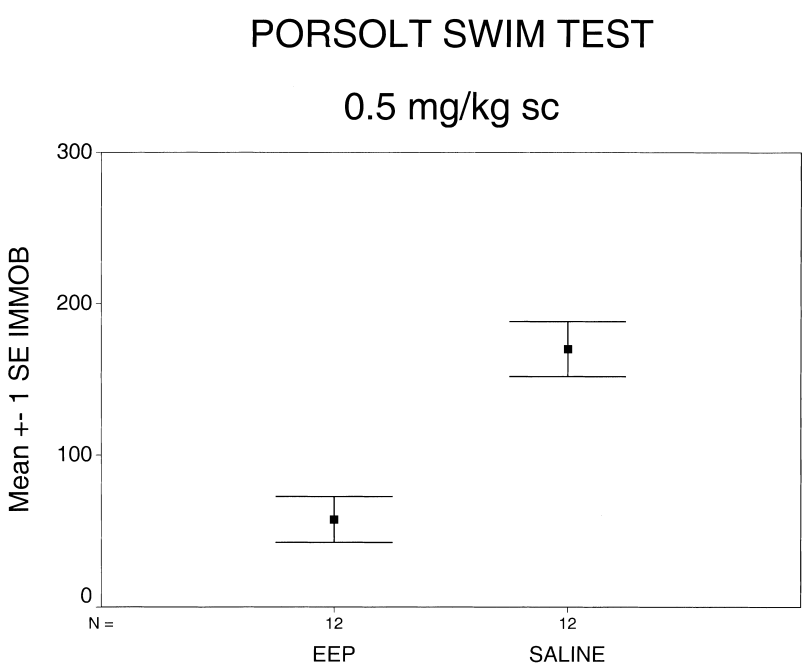

Fig. 3. Mean immobility times in the Porsolt test for animals injected with saline or EEP (0.5 mg/kg, sc) in Experiment I.

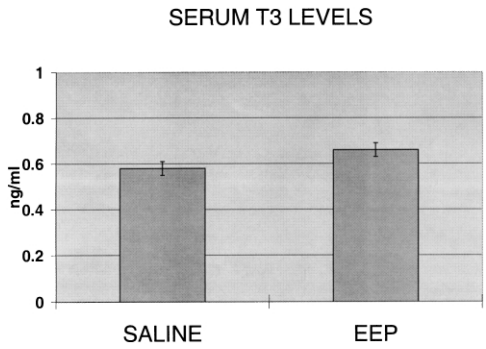

Fig. 4. Mean levels of triiodothyronine in serum taken from animals run in the Porsolt test following injections of saline or EEP (0.5 mg/kg, sc) in Experiment I.

on ice and homogenized in methanol. These homogenates were then centrifuged (1800 rpm, 15 min) and the supernatant decanted, dried completely, and stored at $-70°C$. The samples were reconstituted with 1.0 ml 0.02% NaN_3 prior to radioimmunoassay (RIA).

The first experiment compared the effects of EEP (0.5 mg/kg, sc, dissolved in saline) to animals injected with saline. The second experiment compared an equivalent dose of EFP (0.5 mg/kg, sc, dissolved in saline) to saline. In a final experiment, the behavioral effects of a much smaller dose of EEP (0.05 mg/kg), intraperitoneally injected, was examined, which matched the dose and route of administration originally reported by Mabrouk and Bennett (Mabrouk and Bennett, 1997). In each experiment, two shipments of animals were used. The data from each experiment were therefore analyzed with a randomized block, two-way analysis of variance, with treatment as one factor and block (shipment) as the other factor.

2.3. Immunization procedure

EEP was conjugated to keyhole limpet hemocyanin (KLH, Sigma, St. Louis, MO, USA) by the carbodiimide method (Abraham and Grover, 1971). Four female New Zealand white rabbits (Universal Animal Care, Bloomington, CA) were immunized subcutaneously with a stable emulsion prepared from equal volumes of conjugate (1.5 mg/ml saline) and Freund's complete adjuvant. After shaving a 10×10 cm area of the back or flank, rabbits received 10–12 subcutaneous injections, equaling 1.0 ml of emulsion prepared with the EEP–KLH conjugate. Three weeks later, all rabbits received injections of immunogen identical to that received initially. The rabbits were bled from an ear vein prior to the second and subsequent immunizations. All assays were carried out using EEP antibody 580B4.

2.4. Tissue analysis

T_3 levels in serum were assessed using an Abbott Im_x System. Brain homogenates were assessed for levels of EEP and EFP using EEP antiserum 580B4 (100 μl) diluted 1/475 (final dilution) and EEP and EFP standards, respectively. Levels of TRH were measured with the highly specific TRH RIA. TRH was labeled with ^{125}I using the lactoperoxidase method (Pekary et al., 1983b). Using a small column of 2% BSA-treated Sephadex G10 packed in a 5-ml pipet, the tracer was separated from free ^{125}I by eluting with phosphate-buffered saline (PBS), pH 7.5. It was then diluted in 0.325% normal rabbit serum, 0.025 M EDTA, and 0.02% sodium azide (NaN_3) to 15,000 cpm/0.2 ml. EEP, EFP, and TRH standards and samples were diluted in 0.02% NaN_3. Standards, in duplicate, or samples, in duplicate 100-μl aliquots,

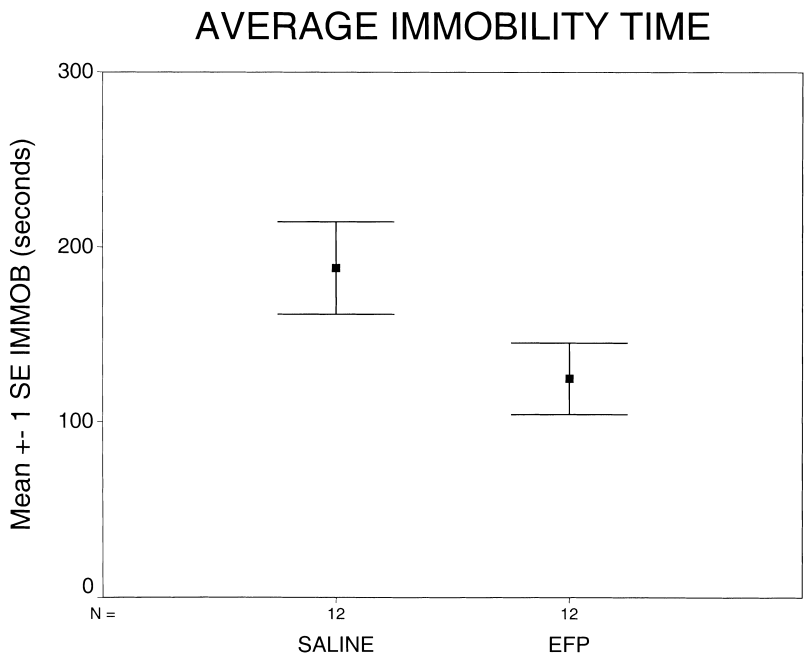

Fig. 5. Mean immobility times in the Porsolt test for animals injected with saline or EFP (0.5 mg/kg, sc) in Experiment II.

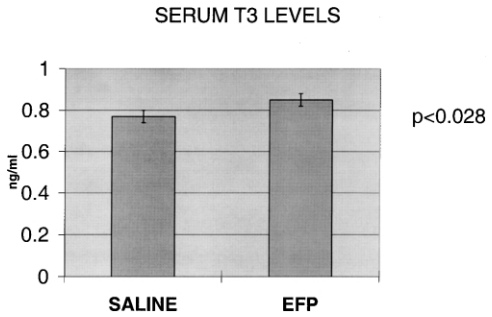

Fig. 6. Mean levels of triiodothyronine in serum taken from animals run in the Porsolt test following injections of saline or EFP (0.5 mg/kg, sc) in Experiment II.

tracer (200 μl), EEP antiserum 580B4 diluted 1:100 in 0.02% NaN_3 in PBS to give 20% specifically bound counts/total counts (B_o/T), and goat antirabbit IgG in 0.02% NaN_3 in PBS (75 μl) were added together, vortex-mixed, and incubated at 4°C for 24 h. Tubes were centrifuged at 1000 × *g* for 30 min, aspirated, and counted for 2 min. The minimum detectable dose was 20 pg/ml. TRH RIAs were performed on the same tissue extracts as previously described (Pekary et al., 1978). All samples from an experiment were run in the same assay. Fig. 1 and Table 1 summarize the relative potency of ^{125}I-TRH displacement in the EEP RIA by TRH, EEP and other TRH-like peptides. The EEP RIA is highly specific for the general structure pGlu-X-Pro-NH_2, where "X" can be any amino acid (Pekary et al., 1999).

2.5. T_3 assay

The normal range for total serum T_3 in young adult male Sprague–Dawley rats, established in our laboratory, is 0.68 ± 0.13 ng/ml (Pekary et al., 1989). The T_3 standard curve is given in Fig. 2.

3. Results

3.1. Experiment I

The mean immobility time of animals injected with 0.5 mg/kg (sc) EEP (58 s, S.E. = 15 s) was significantly less ($F_{1,20} = 20.6$, $P < .001$) than the average immobility time of animals receiving saline (170 s, S.E. = 18 s). No effect of the blocking variable or interaction of the blocking variable with treatment was found (Fig. 3). In addition, an analysis of the T_3 levels of the EEP-injected (0.66 ng/ml, S.E. = 0.04) and saline-injected (0.58 ng/ml, S.E. = 0.03) animals failed to find a significant difference (Fig. 4). These T_3 levels were within the normal range for male rat Serum T_3 established for our laboratory, suggesting that any chilling of the rats by their towel-dried, but damp, fur prior to decapitation did not activate the hypothalamic–pituitary–thyroid axis. Tissue analysis failed to find any changes in TRH or EEP in any of the brain regions analyzed (data not shown).

3.2. Experiment II

The difference in mean immobility time for the 0.5 mg/kg (sc) EFP-injected (125 s, S.E. = 20 s) and saline-injected (188 s, S.E. = 26 s) animals was smaller and failed to reach two-tail significance ($F_{1,20} = 3.9$, $P < .086$). As with the EEP experiment, there was no blocking effect and no interaction

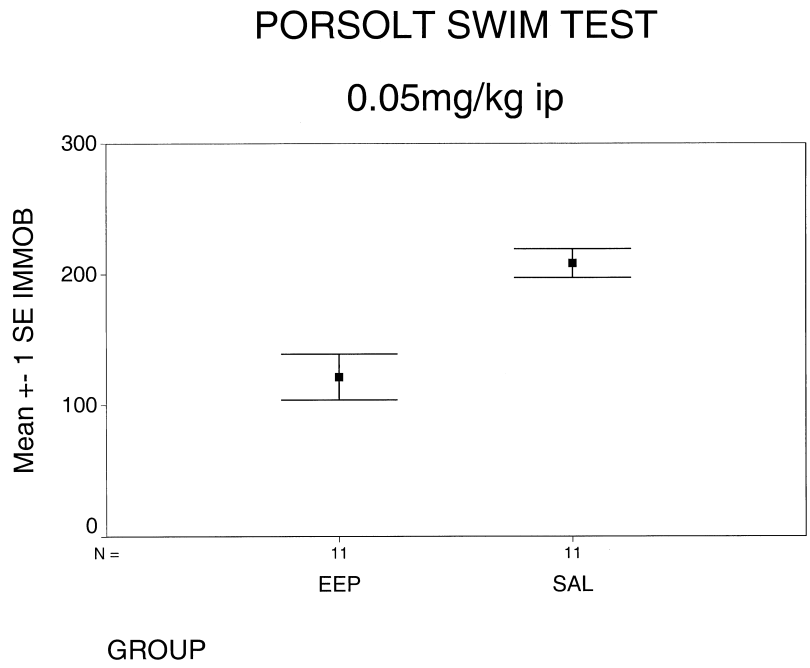

Fig. 7. Mean immobility times in the Porsolt test for animals injected with saline or EEP (0.05 mg/kg, ip) in Experiment III.

of the blocking variable with the treatment variable (Fig. 5). Nevertheless, a significant ($P < .028$, one-tailed) increase in the level of T_3 (Fig. 6) was found in the EFP-injected animals (0.85 ng/ml, S.E. = 0.03) as compared to controls (0.77 ng/ml, S.E. = 0.03). Tissue analysis failed to find any changes in TRH or EFP in any of the brain regions analyzed (data not shown).

3.3. Experiment III

The mean immobility time of animals injected with 0.05 mg/kg (ip) EEP (121 s, S.E. = 18 s) was significantly less ($F_{1,18} = 15.8$, $P < .001$) than the average immobility time of animals receiving saline (208 s, S.E. = 11 s). No effect of the blocking variable or interaction of the blocking variable with treatment was found (Fig. 7). Because no effect upon T_3 levels was observed in the first experiment, where a tenfold increase in EEP was administered, no analysis of T_3 was undertaken in this experiment.

4. Discussion

Injections of EEP produced a 66% reduction in immobility while EFP had no significant effect. The difference in mean immobility time between the EFP- and saline-injected animals was smaller (39% reduction), but it is possible that a larger sample of animals may have demonstrated a significant reduction in immobility with EFP. The variability within the EFP-injected animals was greater that that observed in the EEP-injected animals, suggesting the possibility of a threshold level effect. In addition, the variability among the saline-injected animals in the EFP experiment was greater than that found in the control group in the EEP experiment, thus, making it harder to demonstrate a significant reduction in immobility with EFP.

Whether EFP has a weaker effect upon immobility, or no effect upon immobility at all, the two experiments, taken together, suggest that there is an appreciable degree of sequence specificity in the ability of tripeptide analogues of TRH to reduce immobility time.

Another question is whether the reduction in immobility time found with EEP was secondary to stimulation of thyroid activity. The difference in T_3 levels in the EEP- and saline-injected animals was not significant, but the mean level was, nevertheless, higher in the EEP-injected group. However, EEP does not crossreact with the pituitary TRH receptor (Hinkle et al., 1974). In addition, EFP, which did not produce either a large or significant reduction in immobility time did, in fact, produce a significant increase in T_3 levels, relative to controls. The relative potency for EFP crossreactivity with the pituitary TRH receptor is 6% (Pekary et al., 1983a). Thus, across experiments, there was a dissociation between the effects upon immobility and upon T_3 levels of these two compounds. This suggests that the reduction in immobility found with EEP was not due to stimulation of thyroid function.

Another concern has to do with the report of potential dopaminergic properties of EEP (Mabrouk and Bennett, 1997). Enhanced dopaminergic activity may have antidepressant effects and may be involved in the clinical effects of some antidepressants such as bupropion and nomifensin. However, any nonspecific locomotor effects of EEP, whether dopaminergic or otherwise, may compromise the validity of the behavioral assay used in this experiment. The dose of EEP used in this experiment was reported to increase locomotor activity by 41% across a 90-min interval, as measured by photobeam crossings, and the locomotor effects of higher doses of EEP were found to be significantly inhibited by pretreatment with haloperidol, but there was no description of the time course of this effect (Mabrouk and Bennett, 1997). Although measured for 90 min, all of the difference in activity may have occurred in the early minutes after injection. This is of importance given that the behavioral measure used in the present study was made 1 h after the last injection. In addition, the reversal of the locomotor effects of EEP by haloperidol may have been nonspecific, i.e., haloperidol alone might have retarded locomotor activity.

These same authors reported that EEP, as well as TRH, produced stereotypic behavior across a 45-min interval. These behaviors included forepaw licking and wet-dog shakes, but the magnitude and time course of these effects are not reported, and the behaviors were not analyzed separately. Haloperidol pretreatment was reported to inhibit forepaw licking but not the wet-dog shakes. Again, whether the interaction with haloperidol is specific or nonspecific is difficult to determine.

The results of this study are consistent with the observation that animals receiving ECS demonstrate both a reduction in immobility, in the Porsolt Swim Test, and an elevation in concentrations of EEP in the limbic, but not the striatal, tissue. (In the same tissues, elevations of the same magnitude were found in the concentrations of TRH.) Furthermore, the concentration of EEP measured in hippocampus had a significant negative correlation ($r = -.85$) with immobility in the Porsolt Swim Test. The strength of this relationship is comparable to that found between immobility and concentrations of TRH ($r = -.78$), measured in the same tissue. The similarities in the elevations in concentrations and in the strength of the correlations with immobility are remarkable, given that the two peptides have different precursors (Pekary et al., 1999).

The question then arises as to whether the increase in either of these two peptides is causally related to the reduction in immobility. Indeed, many biochemical changes are induced by ECS, any of which could be merely incidental to the effects of ECS upon immobility. Furthermore, strong correlations between the concentrations of various substances (which are elevated by ECS) within the limbic structures and behavior may merely reflect

differences among subjects in the percentage of current passing through the brain, as opposed to other, surrounding tissues. Thus, the two effects of ECS may become highly correlated, but remain causally unrelated. The present study, which directly and specifically manipulates EEP tends to validate the observations of the effects of ECS on EEP and immobility. The participation of TRH in the antidepressant effects of ECS/ECT are not, however, ruled out by the present data.

Failure to detect an increase in brain EEP–IR about 1 h following the last subcutaneous injection of EEP or EFP may be due to the kinetics of transport of EEP (or EFP) from the subcutaneous injection site into the circulation and across the blood–brain barrier, which may be very slow. For example, an intraperitoneal injection of 1.0 mg/kg EEP requires about 5 h to reach peak levels in various brain regions, which includes a 2-h delay to enter the cerebrospinal fluid (Pekary et al., 1999). A failure to detect an increase in brain EEP–IR may also be due, in part, to the fact that subcutaneous tissues, such as seminal fluid, may contain enzymes that can degrade EEP (and EFP), thereby reducing the amount of peptide that is eventually taken up by brain tissue (Cockle et al., 1994).

The pharmacokinetic half-life of EEP is much longer than that of TRH (Pekary et al., 1999), because EEP is not degraded by the enzymes that rapidly degrade TRH (Heuer et al., 1998; Schomburg et al., 1999). If EEP proves to have either independent antidepressant properties, or the ability to modulate the antidepressant effects of other agents, its manipulation, clinically, may prove to be more efficacious than the manipulation of TRH, which has been reported to produce only transient relief from depression (Callahan et al., 1997; Kastin et al., 1972; Marangell et al., 1994, 1997; Post et al., 1989).

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