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THYROTROPIN-RELEASING HORMONE (TRH) ANTAGONISM OF PENTOBARBITAL NARCOSIS: CENTRAL NEUROMODULATION

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THYROTROPIN-RELEASING HORMONE (TRH) ANTAGONISM OF PENTOBARBITAL NARCOSIS: CENTRAL NEUROMODULATION

by

MICHAEL DAVID HIRSCH

A dissertation submitted to the Graduate Faculty in Neuropsychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

THYROTROPIN-RELEASING HORMONE (TRH) ANTAGONISM OF PENTOBARBITAL NARCOSIS: CENTRAL NEUROMODULATION

by

Michael David Hirsch

Adviser: Professor Walter B. Essman

A series of eight experiments were undertaken to characterize the possible central neuromodulatory mechanisms underlying thyrotropin releasing hormone (TRH) antagonism of barbiturate-induced narcosis in the CF-1_S mouse brain on five levels: (a) physico-chemical, (b) neurochemical, (c) physiological, (d) psychopharmacological, and (e) behavioral. These experiments consisted of studies utilizing behavioral pharmacology, biochemical assays, *in vitro* competitive displacement [^{3}H]-TRH radioreceptor assays, and *in vivo* radiochemical assays. The present results support the findings of other workers that TRH is efficacious both *in vivo* and *in vitro* in the antagonism of central barbiturate actions. Dose-response effects were found in this paradigm for two month old mice but not in three month olds.

This significant age-related difference in the TRH effect appears to be related to ontogenetic differences in hepatic drug metabolism since the biochemical results

indicated that there are significant age-related differences in mouse brain concentrations of pentobarbital following intraperitoneal administrations of the drug. It can be assumed that TRH does not appear to alter hepatic metabolism of barbiturates in these experiments since brain levels of the administered drug are unaltered by central peptide vs. physiological saline injections. The central administrations were effected by a new intraventricular (i.vt.) technique developed for this procedure.

The interinjection duration (IID) between TRH and pentobarbital administrations appears to be an important variable influencing behavioral outcomes in the analeptic paradigm: Delivery of drug combinations in close temporal proximity induces optimal analeptic and nonspecific effects. Several proposed models--age-related, toxic-lethal, locomotor activity, spare receptor, and cooperativity phenomena--have been advanced to explain the interaction of these two effects.

The results also indicate no cyclical diurnal variations in TRH's analeptic actions, an expected result since central peptide activity has been previously found to be independent of pituitary functions.

The radioreceptor and radiochemical results also support the findings of other investigators as well as the present behavioral pharmacology findings: Three barbiturate analogues--phenobarbital, pentobarbital, and thiopental--appear to be effective competitive inhibitors of [^{3}H]-TRH in

the radioreceptor assay. This assay also indicates that there are significant regional differences in the degree of barbiturate inhibition at presynaptic vs. postsynaptic specific high-affinity TRH binding sites. The radiochemical data closely matches the radioreceptor data and indicate that presynaptic and postsynaptic TRH receptors of the limbic forebrain region are strongly implicated in the TRH analeptic mechanism.

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INTRODUCTION

General Implications

This project was conducted to study possible central neuromodulatory mechanisms underlying thyrotropin releasing hormone (TRH) antagonism of barbiturate-induced narcosis in the mouse. Of the several known characteristics of this endogenous hypothalamic polypeptide hormone (or "factor"), this specific behavioral effect is one of the most potent and reliable cited (Prange, Nemeroff, Lipton, Breese, & Wilson, 1978a; Prange, Nemeroff, & Loosen, 1978b). In addition to mice, this analeptic effect has been demonstrated in several other species. However, to date, no one has yet clearly identified, characterized, and localized the central receptor pharmacokinetic activity involved. Therefore, the following drug-receptor paradigm was designed to integrate five levels of information: (a) physico-chemical, (b) neurochemical, (c) physiological, (d) psychopharmacological, and (e) behavioral.

In general, features of the present project crossed several lines of investigation to explain central mechanisms for TRH activities, barbiturate anesthesia, as well as TRH-barbiturate interactions. The data accumulated thus far have permitted the characterization of various TRH- and barbiturate-induced alterations of neuronal excitability and transmission. A number of theories have been proffered to integrate the vast body of data accrued. However, to date, no direct experimental evidence has been provided to directly

validate these theories, such that the information thus far amassed remains as an aggregation of unrelated facts. Hence, the series of experiments presented in this dissertation were designed to provide additional information to elucidate the central mechanism of the TRH antagonism of barbiturate narcosis. This introduction will serve to outline the salient features of relevant background information. A number of related hypotheses will then be proposed apropos to this "central mechanism". The Discussion section will be concerned with further explication of the current findings in the general framework of the relationship between TRH analeptic actions and barbiturate anesthesia.

Both the strength and significance of the present project are based upon several important parallel paradigms currently under investigation: These all have as their common focus the elucidation of central functions for endogenous polypeptides. Impetus for this topical interdisciplinary neuroscience thrust relates to the recent finding that hypothalamo-adenohypophyseal oligopeptide hormones ("factors") act in the central nervous system (CNS) quite differently from their traditional endocrine-regulatory functions (Barbeau, Gonce, & Kastin, 1976; Cooper, Bloom, & Roth, 1978; Guillemin, 1978; Harris, Reed, & Fawcett, 1966; Hughes, 1978; Martin, Renaud, & Brazeau, 1975; Sandman, Miller, & Kastin, 1976; Vale & Rivier, 1975; Vale, Rivier, & Brown, 1977). Heightened interest has been kindled by three major areas of research:

1. It has recently been found that five endogenous peptides--Met-and Leu-enkephalin, and α-, β-, and γ- endorphin--have specific receptors, and *in vivo* and *in vitro* "morphine-like" biological properties in the CNS (Guillemin, Ling, & Burgus, 1976; Hughes, 1975; Hughes, Smith, Kosterlitz, Fothergill, Morgan, & Morris, 1975; Lazarus, Ling, & Guillemin, 1976). Their amino acid sequences have been characterized as fragments of the larger β-lipotropin (β-LPH) pituitary hormone. Radioreceptor and radioimmunoassay (RIA) results reveal that the enkephalins are widely distributed throughout the CNS in high concentrations and have been located within the cerebral cortex, caudate nucleus, hypothalamus, thalamus, and posterior pituitary (Pollard, Llorens-Cortes, & Schwartz, 1977; Simantov & Snyder, 1976; Snyder, Uhl, & Kuhar, 1978). Enkephalins have a high affinity for brain opiate receptors and a low affinity for neurohypophyseal receptors; β-endorphin displays a reversed affinity relationship in this regard (Simantov & Snyder, 1977). Endogenous and synthetic opioid peptides have been implicated in direct neuromodulation (Barker, Neale, Smith, & MacDonald, 1978), as well as in the central modulation of analgesia (Bloom, Segal, Ling, & Guillemin, 1976; Haigler & Spring, 1978; Jacquet & Marks, 1976; Pert, Simantov, & Snyder, 1977); sedation, immobility, hyperactivity-hyperreactivity, and catalepsy (Bloom et al., 1976; Browne & Segal, 1980; Chang, Fong, A. Pert, & C. B. Pert, 1976; Goldstein, Tachibana, Lowney, Hunkapiller, & Hood, 1979;

Haigler & Spring, 1978; Jacquet & Marks, 1976; Pert et al., 1977); learning and memory (Rigter, 1978); altered thermoregulation (Bloom et al., 1976); altered electrical self-stimulation activities (Van Ree & Otte, 1980); altered eating and grooming activities (Katz, 1980); and the attenuation of nociceptive responses during pregnancy (Gintzler, 1980). These endogenous opioids are highly active in the opiate-twitch inhibition bioassay as well as in the [^{3}H]-etorphine radioreceptor assay (Chang et al., 1976; Goldstein, 1976); there is a very high correlation between opioid peptide-induced analgesia and activities in both the bioassay (Kosterlitz & Waterfield, 1975) and the radioreceptor assay (Creese & Snyder, 1975; Chang et al., 1976). It also appears that the brain has a potent neuromodulatory mechanism for controlling central opioid actions (Jacquet, Klee, & Smyth, 1978).

2. The second major area of neuropeptide research stems from the recent discovery that structural modifications of amino acid sequences in parent and fragmentary analogues of adrenocorticotropic hormone (ACTH) and α- and β-melanocyte stimulating hormone (MSH)--structurally related to β-LPH--can lead to profound behavioral changes independent of classical endocrine actions (De Wied, 1974; Van Riezen, Rigter, & De Wied, 1977). Recent findings suggest that a vascular route conveys ACTH from the pituitary directly to the brain (Bergland, Blume, Hamilton, Monica, & Paterson, 1980). Autoradiographic studies of the regional

peptide distribution in the rat brain following intraventricular (i.vt.) [^{3}H]-ACTH$_{4-9}$ administrations reveal that these peptides may be acting preferentially upon a morphologically and functionally distinct class of brain neurons: five minutes after administration, labelled cells were identified in the choroid plexus of the lateral ventricles, lateral septal nucleus, caudate-putamen region, stria terminalis, and cerebral cortex near the cannula tract (Rees, Verhoef, Witter, Gispen, & De Wied, 1980); thirty minutes after administration, other areas were involved, including the hypothalamus, thalamus, fornix, hippocampus, diagonal band of Broca, and habenular. ACTH, MSH, and related structural analogues can restore impaired acquisition of shuttle box avoidance behavior in hypophysectomized rats (De Wied, 1974). The behaviorally active core of ACTH is located in the N-terminal amino acid sequence--ACTH$_{4-10}$--which is also present in α-MSH, β-MSH, and β-LPH (De Wied, 1974, 1978; Urban & De Wied, 1976; Van Riezen et al., 1977). The general findings implicate ACTH and structural analogues in motivational processes, learning, and memory retrieval: Administration of these neuropeptides to animals inhibits extinction of shuttle box and passive avoidance responses, and food- and sex- motivated behaviors (De Wied, 1974; Flood, Jarvik, Bennett, & Orme, 1976; Rigter, Janssens-Elbertse, & Van Riezen, 1976; Urban & De Wied, 1976; Wimersma Greidanus & De Wied, 1976; Van Riezen et al., 1977). Low parenteral doses of ACTH$_{4-10}$ to sham operated rats delay

extinction of a conditioned avoidance response; however, higher doses are ineffective in this paradigm when administered to rats bearing extensive bilateral antero-dorsal hippocampal lesions. This suggests that intact limbic structures--including the hippocampus, septum, and thalamic reticular nucleus--are required for neuropeptides to be efficacious (Wimersma Greidanus & De Wied, 1976). It has also been reported that parenteral administrations of ACTH/MSH_{4-10} profoundly enhance attention, visual-motor learning, and visual memory in normal humans (Miller, Harris, Van Riezen, & Kastin, 1976).

3. Endogenous and synthetic polypeptide analogues are currently being investigated as both adjuvants for clinical psychopharmacological treatment regimes as well as being possible etiological factors in psychopathological states: TRH produces an immediate antidepressant effect in depressive patients (Ehrensing & Kastin, 1976; Prange & Wilson, 1972). This peptide is also an effective potentiator in the L-Dopa (3,4-dihydroxyphenylalanine) plus pargyline drug screening test (Plotnikoff, Prange, Breese, Anderson, & Wilson, 1972); and it also antagonizes α-methyl-p-tyrosine (AMPT, a tyrosine-hydroxylase inhibitor) induced depressions of spontaneous locomotor and conditioned avoidance activities in rats (Kulig, 1975). It has also been reported that the peptide reduces schizophrenics' hallucinations while improving their general psychiatric profiles (Wilson, Lara, & Prange, 1973). It has been suggested that the endogenous

opioid peptides play an important etiological role in mental illness through their actions upon homeostatic regulatory functions (Bloom et al., 1976), and through an induction of catatonia (Bloom et al., 1976; Jacquet & Marks, 1976). Roubicek, Krebs, & Poeldinger (1980) reported that parenteral injections of synthetic Met-enkephalin produced transient improvements in the symptomology of endogenous depressives and hebephrenic schizophrenics. Tentative results indicate that acupuncture-induced analgesia may be related to endorphin activities (Pomeranz, 1977). Ho, Wen, & Ling (1980) reported that heroin addicts have diminished β-endorphin levels, suggestive of disrupted pituitary endorphin systems in these patients. $ACTH_{4-10}$ administrations have been shown to profoundly enhance dimensional attention in mentally retarded subjects (Ferris, Sathananthan, Gershon, Clark & Moshinsky, 1976). It also improves attention in normal subjects (Miller et al., 1976). Clinical studies indicate that this neuropeptide is a relatively safe drug in humans and animals (Van Riezen et al., 1977). Gold, Goodwin, Post, and Robertson (1981) reported that intranasal infusions of a synthetic vasopressin analogue improve cognitive functions and ameliorate the symptomology of depressive patients. Clinical trials indicate that luteinizing hormone-releasing hormone (LHRH) may increase the libidinal drive of hypogonadal men (Mortimer, McNeilly, Fisher, Murray, & Besser,

1974).

Central Focus

TRH Antagonism of Barbiturate Narcosis

Prange, Breese, Cott, Martin, Cooper, Wilson, & Plotnikoff (1974) found that TRH has very potent and significant antagonist capabilities in rodents (mice and rats) for barbiturate-induced narcosis (indices: righting reflex; activity departing a circle) and hypothermia. These findings have been reliably confirmed and further extended (Bissette, Nemeroff, Loosen, Prange, & Lipton, 1976; Breese, Cott, Cooper, Prange, & Lipton, 1974a; Breese, Cott, Cooper, Prange, Lipton, & Plotnikoff, 1975; Breese, Cott, Cooper, Prange, Plotnikoff, & Lipton, 1974b; Cohn, M. L., Cohn, M., Krzysik, & Taylor, 1976; Crowley & Hydinger, 1976, 1977; Holaday, Tseng, Loh, & Li, 1978; Kalivas & Horita, 1979, 1980; Prange et al., 1974; Prange, Breese, Jahnke, Martin, Cooper, Cott, Wilson, Alltop, Lipton, Bissette, Nemeroff, & Loosen, 1975; Prange et al., 1978a, 1978b). The TRH analeptic effect has been found to vary with both TRH and barbiturate dosage levels, with route of TRH administration (intraperitoneal [i.p.], intramuscular [i.m.], intravenous [i.v.], intracisternal [i.c.], intracerebral [i.cb.], and intraventricular [i.vt.]) and with drug order and interinjection durations. This effect is independent of the classical pituitary-thyroid axis (it is unaffected by hypophysectomy, and L-triiodothyronine [T_3] or thyroid-stimulating hormone [TSH, thyrotropin] administrations). The

analeptic effect can also be further dissociated from thermoregulatory actions; it also occurs for a wide range of barbiturates (pentobarbital, phenobarbital, thiopental, secobarbital, and amobarbital), as well as a number of structurally different sedative-hypnotics (chloral hydrate, reserpine, chlorpromazine, diazepam, and ethanol). TRH does not affect barbiturate metabolism ([^{3}H]-pentobarbital disposition) (Breese et al., 1975). The TRH analeptic effect has been further extended to other species (mice, rats, hamsters, gerbils, guinea pigs, rabbits, and monkeys) and appears to be non-sex related (at least in mice receiving i.p. drug administrations of TRH and barbiturate) (Breese et al., 1975; Crowley & Hydinger, 1977; Horita & Carino, 1975).

Critical Issues

There are many important features to the TRH analeptic effect which are poorly understood:

1. Since the effect occurs independent of the pituitary-thyroid axis, and since the effect is most potent with central TRH administration, is this phenomenon CNS mediated?

2. Other hypothalamo-adenohypophyseal polypeptides, amino acid fragments of TRH, TRH metabolites (e.g., deamidated TRH), classical neurotransmitters, and many psychopharmacological agents have all been shown to be mainly ineffective (or their role is unclear) in altering the TRH analeptic effect. In contrast, only TRH and certain (perhaps properly spatially-oriented) structural analogues are effective in producing this effect. Their efficacy appears

to be dissociated from their competitive binding affinities in pituitary TRH receptor preparations and their TSH release potencies in pituitary tissue preparations (Breese et al., 1975; Prange et al., 1975). It is most important to note that competitive radioreceptor TRH binding studies in pituitary tissues reveal highly specific saturable binding, a phenomenon found to be critical to in vivo biological potency--TSH release and prolactin (PRL) release (Burt & Snyder, 1975; Burt & Taylor, 1980; Grant, Vale, & Guillemin, 1973; Vale et al., 1977). Therefore, the present dissertation has considered an analogous relationship between the behavioral aspects of TRH antagonism of barbiturate-induced narcosis and specific competitive radioreceptor TRH binding in CNS tissues.

3. TRH and its effective analogues do not show a dose-response relationship for the analeptic effect after intraperitoneal (i.p.) administration (Breese et al., 1975; Prange et al., 1975). However, this mode of administration can be expected to render the peptide vulnerable to enzyme catabolism (Eskay, Oliver, Warberg, & Porter, 1976). Also, as pointed out by Prange et al. (1974) and directly tested by Stumpf and Sar (1973), only a small amount of parenterally administered TRH reaches the brain compared to the amount that reaches the pituitary. Presumably, TRH and its structural analogues must cross the blood-brain barrier in order to antagonize pentobarbital (Bissette et al., 1976; Prange et al., 1974, 1975). Therefore, the present project

studied this phenomenon using central (i.vt.) administration conditions.

In Hypothesis 1, TRH antagonism of barbiturate narcosis was predicted to show a dose-response relationship when centrally administered because of an elimination of the problems associated with parenteral administrations.

4. TRH-pentobarbital combinations show dose-related toxic and/or lethal effects (Breese et al., 1974b, 1975; Cohn et al., 1976; Crowley & Hydinger, 1976, 1977; Holaday et al., 1978; Prange et al., 1978b).

In addition, as a result of a discrepancy between the biological activity constant of TRH (relative TSH release potency) empirically derived from in vitro secretion studies vs. the binding affinity constant (K_A) found in pituitary receptor experiments (the former constant is 10-fold lower than the latter), a "spare receptor" theory has been advanced (Grant et al., 1973; Vale, Grant, & Guillemin, 1973; Vale & Rivier, 1975): Only a small percentage of receptors need be saturated (occupied) by TRH in order to produce a maximal biological response. Therefore, TRH's "secretory stimulation" curve is shifted to the left of its "binding" curve. Likewise, a "ceiling effect" was also predicted for TRH's analeptic actions: It was predicted that only a small percentage of occupied central TRH receptors would be required for full analeptic actions. Therefore, beyond certain "limits", higher doses of TRH would no longer augment the analeptic action.

It is also important to note that the overall dissociation of TRH away from its pituitary receptor does not follow simple first-order kinetics. The 50% dissociation rate determined by "infinite" dilution in the presence of excess unlabelled TRH is approximately three times more rapid than with "volumetric (no ligand)" dilution alone. In light of "the unusual kinetics of [TRH] binding to its receptor, its dissociation from the receptor, and the slightly different slopes in the competition curves", Vale and Rivier (1975) suggested the phenomenon of "negative cooperativity" as an alternate interpretation of the [^{3}H]-TRH competitive binding data. As De Meyts (1976) pointed out, "negative cooperativity" has several advantages over a "multiple class of [affinity] binding sites" type model: It requires no energy-dependent cell machinery, and requires only a single protein species undergoing conformational changes.

Hypothesis 2 states that higher dose combinations of peptide-barbiturate should yield significant interaction effects and/or are non-dose related as a function of either toxic interactions, or spare receptor phenomena, or negative cooperativity effects.

5. Breese et al. (1975) found that peripherally administered TRH effectively interacted with barbiturate in this analeptic phenomenon up to 30 minutes prior to i.p. pentobarbital administrations, but not at longer interinjection durations. Since the dissociation of TRH away from its binding sites in the cerebral cortex has a half-life of

about 38 minutes (Burt & Snyder, 1975), and since exogenous TRH is enzymatically destroyed in brain homogenates after 30 minutes at 37° C incubations (Winokur, Davis, & Utiger, 1977), exogenous central TRH (i.vt.) administrations would also be expected to significantly decrease after 30-40 minutes. The present project therefore investigated the effects of varying the interinjection duration upon central TRH antagonism of pentobarbital-induced narcosis.

Hypothesis 3 proposed that lower TRH doses were less effective as competitive inhibitors of barbiturates at longer interinjection durations (IID). This was due to peptide dissociation away from the receptor and/or proteolytic degradation prior to barbiturate actions. Therefore, the analeptic actions of lower doses of TRH were also expected to be attenuated at longer IIDs. On the other hand, higher TRH doses were expected to produce either toxic, spare receptor, and/or negative cooperativity phenomena at brief IIDs. At longer IIDs, these phenomena were expected to be attenuated. Thus, higher TRH doses should display greater analeptic actions at longer IIDs.

6. It has been reported that pituitary release of a number of hormones--including TSH, ACTH, growth hormone (GH, somatotropin), prolactin (PRL), luteinizing hormone (LH), follicle-stimulating hormone (FSH)--is subject to diurnal variations (Rubin, R. T., Poland, Rubin, L. E., & Gouin,

1974). Since TRH's analeptic actions occur independent of pituitary-thyroid functions, Hypothesis 4 predicted that these actions would not display diurnal variations.

Background Information

Evidence for TRH Distribution in the CNS

TRH is the first and smallest polypeptide to be isolated, purified, characterized, and synthesized (Vale & Rivier, 1975; Vale et al., 1977); its sequential tripeptide-amino acid structure is L-pyroglutamyl-L-histidyl-L-proline amide (pGlu-His-Pro-NH_2). Synthetic TRH has been shown to stimulate *in vivo* and *in vitro* secretion of TSH from the adenohypophysis in all mammals (including humans). It effects PRL release as well (Brownstein, 1978; Vale & Rivier, 1975; Vale et al., 1977). Brownstein, Palkovits, Saavedra, Bassiri, and Utiger (1974) utilized a micropunch technique on frozen rat brain tissues to obtain discrete nuclei for identification of immunoreactive TRH via RIA. TRH was found in highest concentrations in hypothalamic regions: The median eminence had the highest TRH level (25% of total hypothalamic content) for this brain region which was four-fold higher than for the ventromedial nucleus, the next highest area. High TRH concentrations were also found scattered amongst several other hypothalamic regions, in the following descending order: periventricular, dorsomedial, arcuate, and paraventricular nuclei. The preoptic area contained moderate TRH levels. The septal region also contained high TRH concentrations, especially in

the lateral nucleus, followed by the dorsal nucleus. Thus, endogenous TRH was localized predominantly within the area adjacent to the Ventricle III in the hypothalamic region and adjacent to the lateral ventricle in the septum. It is likely that TRH-barbiturate interactions are mediated within many of these brain regions which are high in endogenous TRH, i.e., in hypothalamic, preoptic, and septal areas. Of course, this would require further demonstration that these regions have physiologically functional sites for this specific phenomenon.

Indirect immunofluorescent microscopy has provided further evidence for TRH localization and extends earlier RIA data for the localization of TRH in the axon nerve terminal (Hökfelt, Fuxe, Johansson, Jeffcoate, & White, 1975); in addition, moderate-high TRH concentrations are found in both central (preoptic, septal, and brainstem motor nuclei) and peripheral (ventral horn of spinal cord) extrahypothalamic regions.

Youngblood, Lipton, and Kizer (1978) separated TRH-like substances from several regions of the rat brain by thin-layer chromatography (TLC). The immunoreactive (RIA) "elution profiles" obtained indicated that the hypothalamic and septal-preoptic regions might contain authentic TRH.

Spindel and Wurtman (1980) fractionated extracts from rat brain homogenate and via either TLC or high-pressure liquid chromatography (HPLC) isolated components from which TRH was identified immunoreactively by RIA. They identified

specific immunoreactive TRH in many brain areas which had chromatographic characteristics that closely corresponded to synthetic TRH. This finding was validated by five different variations of their procedures. They reported finding authentic TRH in the frontal cortex, hypothalamus, septum, preoptic area, corpus striatum, and the brain stem. Spindel and Wurtman suggest that Youngblood et al. may have failed to identify authentic TRH in other brain areas due to poor recoveries from multiple extracts as well as non-specific interfering compounds eluted, problems which they claim to have avoided.

Kubek, Wilber, and George (1979) identified high levels of immunoreactive TRH via RIA in human hypothalamic nuclei and adjacent regions. Highest TRH concentrations were found in the median eminence area, followed by the arcuate nucleus; intermediate concentrations were found in the hypothalamic paraventricular and periventricular nuclei; lowest concentrations were found in the mammillary complex as well as posterior, supraoptic, and anterior hypothalamic nuclei. While the human brain levels were lower than those reported for the rat brain (Brownstein et al., 1974), there was generally a close correspondence in regional brain TRH concentrations between the two species. Kubek et al. (1979) also demonstrated the complete stability of endogenous TRH in the rat brain: Incubations of hypothalamic and cerebral cortex tissue extracts at 4° C for 16 hours did not display altered TRH, nor did intact tissues incubated at 25° C for 1

hour.

Pearse's (1978) amine precursor uptake and decarboxylation theory (APUD) postulates that all peptide-producing cells are derived from the embryonal neuroectoderm. In support of this theory, Leppäluoto, Koivusalo, and Kraama (1978), using RIA techniques, found TRH localized in rat CNS, endocrine, and gastrointestinal (GI) tract tissues.

Evidence for TRH as a Neuromodulator

In addition to its classically characterized adenohypophyseal actions, an extraendocrine central neuromodulatory role has been postulated for this peptide based upon several findings: its widespread and extremely high central concentrations (Brownstein et al., 1974; Hökfelt et al., 1975; Kubek et al., 1979; Leppäluoto et al., 1978; Spindel & Wurtman, 1980; Youngblood et al., 1978); its localization within axon nerve terminals (Hökfelt et al., 1975; Winokur et al., 1977); the observation that microiontophoretic applications can produce altered neuronal spike frequencies (Dyer & Dyball, 1974; Renaud & Martin, 1975; Renaud, Martin, & Brazeau, 1975, 1976; Winokur & Beckman, 1978; Yarborough, 1976, 1978; Yarbrough & Singh, 1978); its central binding distribution (Burt & Snyder, 1975; Burt & Taylor, 1980; Ogawa, Yamawaki, Kuroda, Ofuji, Itoga, & Kito, 1981); the fact that TRH alters neurotransmitter release and turnover in the brain (Heal & Green, 1979; Jessel & Richard, 1977; Keller, Bartholini, & Pletscher, 1974; Malthe-Sørenssen, Wood, Cheney, & Costa, 1978); that it can also be released

from brain-derived synaptosomes in transmitter-like fashion (Schaeffer, Axelrod, & Brownstein, 1977; Warberg, Eskay, Barnea, Reynolds, & Porter, 1977); and its profound pharmacological actions upon behavior (Bissette et al., 1976; Breese et al., 1974a, 1974b, 1975; Cohn et al., 1976; Crowley & Hydinger, 1976, 1977; Ehrensing & Kastin, 1976; Holaday et al., 1978; Kalivas & Horita, 1979, 1980; Kulig, 1975; Poltnikoff et al., 1972; Prange & Wilson, 1972; Prange et al., 1974, 1975, 1978a, 1978b; Wilson et al., 1973).

In order to further verify whether TRH actually does function as a central neurotransmitter-neuromodulator, it would thus seem critical to demonstrate that TRH and some of its structural analogues are capable of specific high-affinity saturable binding at central TRH receptor sites.

Specific Saturable Binding

Burt and Snyder (1975) utilized [^{3}H]-TRH radioreceptor binding studies to investigate specific TRH binding in the rat brain. They found high concentrations of high-affinity TRH binding sites distributed in the cerebral cortex, hypothalamus, hippocampus, and midbrain regions, a finding confirmed and extended in the monkey brain by Ogawa et al. (1981). The affinity constant (K_A; or dissociation constant, $K_D = 1/K_A$) values obtained for the cerebral cortex TRH binding sites were found to be similar to pituitary K_A values. Both types of tissues showed similar, yet distinct, recognition requirements for a number of TRH structural analogues.

A later study by Burt and Taylor (1980), which improved on the earlier technique by utilization of [^{3}H-Me-His^{2}]-TRH in order to discriminate high- vs. low-affinity [^{3}H]-TRH binding sites, indicated that the highest concentration of specific TRH sites in the sheep brain was located in the nucleus accumbens-septal area. This brain region was also found to have distinct structural recognition requirements as compared with anterior pituitary sites. Thus, these findings lend strong support to the high specificity requirements expected for physiologically relevant TRH receptors in the CNS. Second, the results further indicate that brain vs. adenohypophyseal binding site recognition for TRH and its structural analogues can be distinguished in a mode similar to Breese et al's. (1975) and Prange et al's. (1975) reports that analeptic actions of TRH analogues could be dissociated from adenohypophyseal biological activities (TSH release). This latter point will be further explicated in the Discussion section.

For now, the following testable hypotheses can be stated:

Hypothesis 5 proposed that the TRH analeptic mechanism is directly mediated at specific TRH binding sites in the mouse CNS. An important feature of the present study was an in vitro competitive displacement [^{3}H]-TRH radioreceptor assay to test this hypothesis, i.e., to assess whether barbiturates effectively compete with TRH for specific brain binding sites.

Hypothesis 6 maintained that the TRH analeptic effect is not a function of altered barbiturate metabolism. This latter hypothesis was based upon Hypothesis 5, which proposed that the analeptic effect is mediated at specific TRH receptors and, as a result, the effect would not be expected to be a function of altered drug metabolism. To test this hypothesis, a biochemical assay was performed to assess regional brain levels of pentobarbital following TRH-barbiturate administrations vs. saline (control)-barbiturate administrations. In addition, an unexpected "age" factor was observed during the pharmacological studies. Therefore, a second pentobarbital assay was performed to further assess this factor.

Neurochemical-Neuroanatomical Considerations

A central cholinergic mechanism has been implicated in the TRH analeptic effect: Breese et al. (1975) found that centrally administered atropine and its methyl derivative, two specific muscarinic acetylcholine (ACH) receptor antagonists, blocked TRH antagonism of pentobarbital narcosis. Peripheral administrations, on the other hand, failed to produce this effect. Phentolamine, an adrenergic antagonist, was also without effect in this paradigm. These findings agree with the inverse relationship between brain ACH content and neuronal activity levels during altered states of consciousness (i.e., sleep-arousal) following pentobarbital and other anesthetics vs. convulsant drug administrations (Elliott, Swank, & Henderson, 1950; Giarman & Pepeu, 1962;

Phillis, 1968, 1970; Richter & Crossland, 1949; Simon & Kuhar, 1975; Tobias, Lipton, & Lepinat, 1946). It has been suggested that transmitter release and hence subsequent metabolic degradation are under direct regulation by neuronal impulse flow. Consequently, barbiturate-induced depressant influences upon neuronal activities abnormally attenuate ACH turnover and/or augment central reserve pools of transmitter (Atweh, Simon, & Kuhar, 1975; Simon, Atweh, & Kuhar, 1976; Simon & Kuhar, 1975); central stimulatory influences like those of TRH are assumed to effect increased transmitter turnover and/or depletions of their pools (Malthe-Sørenssen et al., 1978).

Further support for a cholinergic mechanism is found in Yarbrough's (1976, 1978) and Yarbrough and Singh's (1978) reports that TRH applications can potentiate the excitatory actions of microiontophoretically applied ACH and carbamycholine (carbachol) in the muscarinically-identified somatosensory cortex of pentobarbital anesthetized rats.

A cholinergic component of the ascending reticular activating system (ARAS), implicated in arousal, has been anatomically identified by experimental and histochemical specific cholinesterase-staining techniques (Lewis & Henderson, 1980; Shute & Lewis, 1963, 1967). The ARAS consists of dorsal and ventral cholinergic tegmental pathways of both mesencephalic reticular formation (MRF) and substantia nigra origins, respectively, which are then relayed to tectal, thalamic, and basal forebrain areas, and then further

projected to the cerebral cortex, and olfactory bulb. These pathways are closely related to the thalamic and extra-thalamic portions of the ARAS. Classical experiments strongly implicate the ARAS in the mediation of altered states of consciousness during normal sleep-wakefulness as well as during stimulatory-inhibitory influences (Bradley, 1958; French & Magoun, 1952; Lindsley, Schreiner, Knowles, & Magoun, 1950; Livingston, 1959; Moruzzi & Magoun, 1949). Both atropine and pentobarbital act within levels of this system (Bradley, 1958; Phillis, 1968). It is thus likely that TRH's antagonistic actions towards barbiturate narcosis are localized within various levels of this arousal system.

It is not clear, however, if TRH-barbiturate interactions influence only the cholinergic components of this arousal system, or whether other non-cholinergic transmitter components may also be involved in the analeptic effect since: (a) non-cholinergic corticopetal fibers of thalamic reticular origin are still capable of inducing cortical electroencephalogram (EEG) desynchronization responses following atropine administrations during reticular stimulations (Shute & Lewis, 1967), (b) monoamine-containing fibers can influence the cortical EEG (Fuxe, 1965), and (c) both TRH and barbiturates influence other transmitter systems of the brain, including norepinephrine (NE; 3,4-dihydroxyphenylethanolamine), dopamine (DA; 3,4-dihydroxyphenylethylamine), serotonin (5HT; 5-hydroxytryptamine); γ-aminobutyric acid (GABA), glutamic acid (Glu; L-glutamate), and, possibly,

glycine (Gly) and histamine (Cott & Engel, 1977; Green & Grahame-Smith, 1974; Heal & Green, 1979; Huidobro-Toro, Scotti de Carolis, & Longo, 1974; Jessel & Richards, 1977; Keller et al., 1974; Lotti, Yarbrough, & Clineschmidt, 1980; Plotnikoff et al., 1972; Renaud et al., 1976; Tabakoff, Yanai, & Ritzmann, 1978; Waller & Richter, 1980; Winokur & Beckman, 1978).

On the other hand, Burt and Snyder (1975) and Burt and Taylor (1980) tested a large series of transmitters and related drugs and found that all agents tested--including carbachol, atropine, d-tubocurarine, and oxotremorine--lacked effective competitive displacement activities in [^{3}H]-TRH radioreceptor assays. Thus, it is likely that TRH's analeptic actions are not directly related to any other putative extra-TRH transmitter receptor actions. Hence, TRH's analeptic effect may be directly related to only a specific TRH binding site-mediated action. Nonetheless, TRH binding sites might still be adjacent to or impinge upon other transmitter systems of the ARAS. Thus, it would seem important to assess whether specific central TRH binding sites reside within various regions innervated by the ARAS.

Additionally, it seems likely that the neuroanatomical focus of the present study must be further expanded: Green (1960) and Green and Arduini (1954) reported that pentobarbital narcosis is accompanied by both neocortical EEG

synchrony as well as asynchronous hippocampal discharges which interrupt the normal "theta" rhythm. It has been suggested that the "theta" rhythm represents hippocampal arousal since: (a) the response occurs following sensory stimulation that also produces behavioral and cortical arousal, (b) the hippocampus receives direct connections from septal, entorhinal, and presubiculum regions of the limbic cortex, and (c) the hippocampus sends efferent fibers to the hypothalamus, the diffuse thalamic projection system, and the brainstem reticular formation (BSRF) (Green, 1960; Green & Arduini, 1954). Therefore, pentobarbital actions may also involve the hippocampus and other interconnected brain regions which include the limbic forebrain and limbic brain stem. This viewpoint would agree with Ranson's (1939) finding that lesions of the mammillary body and posterior hypothalamus--two main projection areas of the hippocampus--produce a profound somnolence, a finding further supported by Nauta's (1946) report that different regions of the hypothalamus are involved in the mediation of arousal-sleep states.

Hence, Green and Arduini (1954) proposed that hippocampal activities can be subsumed under Papez' (1937) theory that the limbic rhinencephalon serves to integrate affective and visceral activities of higher levels of the CNS. Pentobarbital's anesthetic activities appear to involve brain regions within and innervated by the entire Papez limbic circuit: Pentobarbital markedly decreases neuronal

activities in the cerebral cortex, hippocampus, and hypothalamus; septal stimulation significantly reverses these effects (Atweh et al., 1975; Simon et al., 1976; Simon & Kuhar, 1975). It is therefore important to note that both the analeptic actions and specific high-affinity binding sites of TRH have been localized within both forebrain and brain stem areas of the limbic circuit: Kalivas and Horita (1979, 1980) utilized a microinjection technique to administer TRH to discrete neuroanatomical sites in the rat following pentobarbital narcosis. The septal region was found to be the most sensitive brain site for inducing TRH analeptic actions. Other highly sensitive brain sites also included the nucleus accumbens, the medial thalamus, the interpeduncular nucleus, the medial preoptic area, the medial hypothalamus, the diencephalic-mesencephalic periventricular gray regions, and the locus ceruleus. Moderate sensitivities were also reported for the dorsal hippocampus, MRF, the substantia nigra, and the parafascicular nucleus. In this regard, it is significant that Ogawa et al's (1981) [^{3}H]-TRH radioreceptor assay results indicated that the highest levels of specific high-affinity TRH bindng sites of the monkey brain are localized within the limbic hippocampus and amygdala regions; the next highest levels are in the frontal cortex, interpeduncular nucleus, and periaqueductal gray matter of the midbrain. These radioreceptor findings agree with similar reports by Burt and Snyder (1975) and Burt and Taylor (1980). These latter two groups of

investigators found that the nucleus accumbens has the highest concentration of TRH binding sites. Thus the following hypotheses can be proposed:

Hypothesis 7 suggested that if barbiturates behave as effective competitive inhibitors in the [^{3}H]-TRH radioreceptor assay, then these actions must occur within various CNS levels innervated by Papez' limbic circuit. Several regions implicated include the septum, hippocampus, amygdala, hypothalamus, thalamus, and cerebral cortex. The present radioreceptor data were further assessed on two important levels: (a) regional CNS distribution and (b) receptor membrane type. The TRH "receptor" preparations were highly purified presynaptic vs. postsynaptic receptor membranes from different brain areas obtained via refined subcellular fractionation methods. Therefore, the binding data were expected to be highly specific to TRH receptor activities.

Hypothesis 8 suggested that TRH-barbiturate *in vivo* pharmacological interactions occur at specific TRH receptor sites within limbic brain areas. Another important feature of this project was a test to determine whether barbiturates can effect an *in vivo* displacement of centrally diffused [^{3}H]-TRH from the same limbic brain regions as found in the *in vitro* [^{3}H]-TRH radioreceptor assay. This latter test was crucial in order to validate the radioreceptor results. Also, as noted above, a number of brain areas have been implicated in arousal-anesthesia mechanisms, and TRH's analeptic actions in particular. Therefore, the regional brain

distributions found for the present pharmacological and radioreceptor data will be further compared with the data of other investigators.

Summary

To summarize, the present project has focused on the following areas of investigation related to TRH's analeptic actions:

1. Behavioral pharmacology studies to assess TRH-barbiturate dose-response relationships (main vs. interaction effects), as well as the subsequent influences of altered interinjection durations, diurnal variations, and age upon these relationships.

2. Biochemical assays to determine the effects of central TRH administrations and age factors upon regional brain levels of pentobarbital.

3. In vitro competitive displacement [^{3}H]-TRH radioreceptor assays: (a) to identify the regional brain distribution of specific high-affinity (presynaptic vs. postsynaptic receptor membrane) TRH binding sites; (b) to determine whether barbiturates act as competitive inhibitors at specific TRH binding sites.

4. An in vivo radiochemical assay to determine the regional brain distribution of [^{3}H]-TRH-pentobarbital sites of interaction.

The following eight hypotheses were directly tested by these studies:

1. In contrast with parenteral administrations,

central TRH administrations were predicted to display dose-response relationships in the analeptic paradigm.

2. Higher dose combinations of TRH-barbiturate were predicted to yield interaction effects and/or be non-dose related.

3. Both direct analeptic actions as well as indirect drug interaction effects of lower doses of TRH were expected to be attenuated at longer IIDs (in comparison to shorter IIDs), while the actions of higher peptide doses were expected to be either augmented or else less attenuated than lower doses.

4. TRH's analeptic activities were predicted to display a lack of diurnal variability.

5. It was proposed that the TRH analeptic mechanism would be directly mediated at specific TRH binding sites.

6. It was anticipated that TRH would not affect hepatic metabolism of barbiturate. Hence brain levels of barbiturate should thereby remain unaltered by the peptide.

7. Barbiturate analogues were expected to behave in vitro as competitive inhibitors at TRH receptors localized within CNS regions innervated by Papez' limbic circuit.

8. In vivo TRH-barbiturate pharmacological interactions were predicted to occur at specific TRH receptor sites within limbic brain areas.

Clearly, the heuristic value of this research lies in a series of interrelated approaches (across the five previously cited levels) to TRH central neuromodulation and its

antagonism of barbiturate-induced narcosis. From a pragmatic viewpoint, such data could suggest possible clinical approaches to the management of medical (anesthesia) and non-medical (drug abuse) barbiturate overdoses.

METHODS

Experiment 1--Histology

Purpose

This experiment was performed to test the precision of a free-hand unilateral intraventricular (i.vt.; right lateral ventricle) injection technique to be employed in further experiments for the introduction of TRH. The data were used to determine the feasibility of utilizing this procedure in pharmacological studies which require central drug administrations.

Subjects

Twenty male CF-1_S mice (Charles River Breeding Labs), weighing 30-40g and 3 months of age, were used. The mice were maintained on Purina Chow and tap water ad libitum, and were housed 3 animals per cage in quarters kept at constant room temperature (23-25 °C) with alternating 12 hours light/ 12 hours dark periods.

Materials

The dye used to identify central i.vt. injection coordinates was Harris' hematoxylin (pH 6.0). This dye was prepared as a mixture of 1 part hematoxylin, 20 parts aluminum alum, 10 parts absolute alcohol, 200 parts glass-distilled deionized water, 0.5 parts mercuric chloride, and 8.0 ml of glacial acetic acid. 5.0 µl of the dye was administered (i.vt.) with a 10.0 µl capacity (Hamilton Company Model #701-LT) microsyringe mounted with a 30 gauge, 11.6 mm length (Becton Dickinson Model #1256) LNR Yale stainless steel

beveled needle. The needle length was readjusted by a specially designed aluminum stylus and calibrated against a 1.0 mm increment ruler. The ruler was calibrated against a micron caliper and had precision limits of 0.1 mm. All scalp, skull, and brain coordinates were also measured against this ruler.

Procedure

Pilot studies. Injection (i.vt.) coordinates were determined in a pilot study which utilized 60 mice that were matched in age, sex, body weight, and subspecies with the animals in the testing study (histology). Development of precision in the injection technique was effected by: (a) Killing the first set of animals (n = 10) by cervical dislocation, followed by central injections of the dye with the microsyringe apparatus into various sites while utilizing different scalp, skull, and brain coordinates. Surgical procedures consisted of making scalp incisions to identify the relationship between scalp and skull landmarks; splitting of skulls to identify skull and brain coordinate relationships; and removal of skulls and mouse brains followed by coronal brain hemisections to localize dye; (b) Injecting a second set (n = 20) of animals while alive, alert, and mobile in order to develop greater precision in the actual i.vt. procedure; (c) Injecting a third set (n = 20) of live and mobile mice, followed by killing and surgery to check the absolute precision of the procedure against the determined scalp, skull, and brain coordinates; (d) Injecting a

fourth set (n = 10) of live mice followed by killing, surgery, coronal hemisectioning, and histology to make final determinations of all coordinates.

Testing (histology). The precision of the central administration technique was then evaluated: Twenty mice matched by subspecies, age, sex (all male), and body weight with the pilot study animals were injected (i.vt.) with dye from the microsyringe apparatus by utilizing the same previously determined scalp coordinates. When a mouse was ready to be centrally injected, it was isolated in a separate standard wire cage for 10 min. to adapt the animal. The mouse was then picked up by its tail and placed atop the closed cage lid. The mouse's tail was then firmly grasped in the right hand and tautly pulled backwards. Next, the skin at the nape of the neck was grasped firmly with the experimenter's left fingers. At this point, the tail was transferred to the left palm and fifth digit, where it was held tautly. The animal was then firmly placed on the experimental table with all its limbs forced into a flexed position, and with the surface of its head placed parallel to the plane of the table. The skin of the neck was allowed some slack to guarantee that the animal could breathe properly. This procedure thus placed the mouse's head in a stable and properly aligned position for the subsequent i.vt. dye administration, which was effected by holding the microsyringe apparatus in the right hand. The needle had been calibrated against the ruler prior to this time by

adjusting the stylus. Viewing the needle length (which, in this case, was 4.5 mm after adjustment) allowed for precise estimations of scalp injection coordinates relative to scalp landmarks (see Results). The needle was then carefully placed perpendicular to the mouse's scalp and slowly lowered through the skull until fully inserted. The right hand rapidly slid along the barrel of the microsyringe and the plunger was gradually and completely depressed, thereby ejecting dye into the mouse's brain.

Beginning at 9 a.m., live and mobile mice were successively injected (i.vt.) every 10-15 min. over two separate daily sessions. At 3 min. post-injection, a given animal was killed by cervical dislocation. Scalp coordinates were measured by ruler and later statistically averaged across animals. Midsagittal and lateral scalpel incisions of the scalp were made, and the skull was scraped with a dull scalpel and rinsed with physiological (0.9%) saline. The skull coordinates were then measured relative to bregma, lambda, and the midsagittal suture skull landmarks by utilizing the calibrated ruler. Mean and standard error values were calculated for skull coordinates. The skull was then cracked with a rongeur and removed with a pair of curved forceps. The brain was removed with a spoon-shaped spatula and mosquito forceps, and then immersed in a 10% formalin solution for one week.

The mouse brains were subsequently frozen in a stainless steel well filled with a mixture of 95% alcohol and dry

ice. Coronal brain sections (10 μm thick) were then cut on a microtome. Starting at the site of injection, successive coronal sections (every 5th or 10th slice) were mounted on glass slides coated with 0.5% gelatin solution. They were then dehydrated in absolute alcohol and cleared with several changes of xylene. They were fixed with Canadian balsam and left uncovered to allow for greater viewing visibility.

Photomicrographs of the mounted tissues viewed under a light microscope (10 x ocular power) were then obtained through an Olympus research microscope with photomicrography attachment to further identify and document the brain site of injected dye. The film utilized was 35 mm Kodak Professional Ektachrome (EPY 135-36) which was later processed (Kodak Company) for 35 mm transparencies. The precision of the i.vt. procedure was determined by viewing slides of the mounted tissues under a light microscope (Bausch-Lomb) at 1-10 x ocular power. The criterion of precision was defined by predominant localization of identified violet dye within the right lateral ventricle of the mouse brain. A precision value was calculated based upon the percentage of brains (out of a total of 20 brains) meeting this criterion. Several color photographic prints (5 in. x 3.5 in. [0.13m x 0.09m]) were then obtained (Kodak Company) from the color transparencies as representative of the general histological findings. These prints are presented in RESULTS along with presentation of the precision determinations for the central (i.vt.) administration technique.

Experiment 2--Behavioral Pharmacology 1

Purpose

This experiment was performed to determine dose levels of TRH necessary to effectively antagonize pentobarbital-induced narcosis. Dose-response effects were predicted for both drugs below critical limits. Beyond these limits, the two drugs were expected to have significant interaction effects which would disrupt the dose-response relationship.

Subjects

Male CF-1_S mice (Charles River Breeding Labs), three months of age and weighing 30-40g, were used. The animals were maintained on tap water and Purina Chow ad libitum, and were housed 3 per cage in quarters kept at constant room temperature (23-25 °C), with alternating 12 hours light/12 hours dark periods. A total of 90 animals were randomly assigned to given identification codes (metal ear tags; National Band and Tag Co.), litter groups and housing cages, drug treatment subgroups, and time of experimentation. Prior to experimentation, animals were allowed to sufficiently adapt to housing and general laboratory environments in order to eliminate nonspecific stress.

Materials

Crystalline TRH (molecular weight 362.4; Sigma Chemical Company) was dissolved in physiological (0.9% NaCl) saline vehicle, distributed into 50-100 µl aliquots of 0.2% or 0.4% (W/V) TRH solutions, and stored in a freezer (-10 °C) until utilized. Before each experimental session, frozen

TRH aliquots were thawed for several minutes at room temperature (23-25 °C), and then stored in a refrigerator (0 °C) between injection periods. A 100 µl volume of saline vehicle was also refrigerated between administration periods. Fresh solutions of sodium pentobarbital (molecular weight 248.3) were prepared before each session, and refrigerated between administrations along with saline vehicle. The pentobarbital solutions were 0.5% and 0.7% (W/V). Microsyringe equipment was utilized for i.vt. procedures as described in Methods--Experiment 1. Plastic 1 ml tuberculin syringes mounted with disposable stainless steel 25 gauge, 5/8 in. (0.02m) needles (Scientific Products) were used for pentobarbital i.p. administrations.

Procedure

A 3 X 3 factorial design was provided to analyze main and interaction effects of different dose combinations of TRH and pentobarbital. The nine levels of treatment assignments are outlined in Table 1 (Cohen, 1977; Kirk, 1968; Winer, 1971). Sample sizes were determined by the power analysis procedures of Cohen (1977) and Kirk (1968).

All mice were weighed prior to testing. Using a Hamilton Microsyringe mounted with a 30 gauge, 4.5 mm adjusted length LNR Yale needle, either 10 µg TRH, 20 µg TRH, or saline in 5 µl sterile physiological saline vehicle was injected unilaterally into the right lateral ventricle as described in Experiment 1 (Methods). An independent histological study previously confirmed the precision of the

Table 1

Drug Treatment Assignments

Via a 3 X 3 Factorial Design

		TRH Levels (i.vt.)		
		0 µg	10 µg	20 µg
Pento-barbital Levels (i.p.)	0 mg/Kg	Group I (n = 10)	Group II (n = 10)	Group III (n = 10)
	50 mg/Kg	Group IV (n = 10)	Group V (n = 10)	Group VI (n = 10)
	70 mg/Kg	Group VII (n = 10)	Group VIII (n = 10)	Group IX (n = 10)

i.vt. procedure (see Results, Experiment 1). All central injections were followed 1 minute later by an injection (i.p.) of either 50 mg/Kg pentobarbital, 70 mg/Kg pentobarbital, or saline in sterile saline vehicle (volume adjusted to 1.0% of body weight). Starting at 9:00 a.m., 1 mouse from each subgroup was successively administered an assigned drug treatment combination in randomly assigned order over a 5-6 hr. period. Animals were tested in separate metal cages containing sawdust bedding to minimize hypothermia. Pentobarbital-induced narcosis was measured. Narcosis was defined as the inability of an animal to right itself 3 times/min. after being placed on its back and was timed by a stop watch. These experimental procedures were replicated over 10 matched sessions.

Experiment 3--Behavioral Pharmacology 2

Purpose

This experiment was performed to further assess TRH dose-response relationships in the antagonism of pentobarbital-induced narcosis. Other relevant independent variables were simultaneously manipulated. Interinjection durations (IIDs) were varied to establish the time course of TRH interactions with barbiturates. The order of drug conditions was altered to determine if sequence effects were important in drug administrations. In addition, TRH actions were studied at different time periods to assess diurnal variations in the analeptic effect.

Subjects

Male CF-1_S mice (Charles River Breeding Labs), two months of age and weighing 25-38g, were used. The animals were handled, housed, and fed in a manner similar to Experiment 2. A total of 60 mice were randomly assigned to treatment subgroups.

Materials

The same materials were used in Experiment 3 as were used in Experiment 2.

Procedure

A split-plot (repeated measures) factorial design was utilized. The random assignment of mice is outlined in Table 2. A total of 120 observations were obtained. The same general drug handling and administration techniques used in this experiment have already been outlined in

Table 2

Random Assignment of 2 Month Old Male Mice

to Repeated Measures Treatment Groups

(Split Plot Factorial Design): Experiment 3

TRH (i.vt.) Groups	n	Order of Interinjection Durations (IID) (Counterbalanced)	
		Session 1	Session 2
		A. M. Groups:	
0 µg	5	Short	Long
	5	Long	Short
10 µg	5	Short	Long
	5	Long	Short
20 µg	5	Short	Long
	5	Long	Short
		P. M. Groups:	
0 µg	5	Short	Long
	5	Long	Short
10 µg	5	Short	Short
	5	Long	Long
20 µg	5	Short	Short
	5	Long	Long

Note. All assignments randomized and balanced within and across sessions; short IID = TRH at 1 min. before 50 mg/Kg pentobarbital (parameter), long IID = TRH at 45 min. before 50 mg/KG pentobarbital.

Experiments 1 and 2. However, a 50 mg/Kg sodium pentobarbital administration level was maintained throughout the experiment. The barbiturate was injected (i.p.) as a 0.5% (W/V) solution with a volume adjusted to 1% of body weight. Central (i.vt.) TRH dose levels were 0 µg (physiological saline), 10 µg, or 20 µg in a 5 µl vehicle volume. Two different interinjection durations--1 min. (short) vs. 45 min. (long) before pentobarbital--were manipulated within drug treatment subgroups in a counter-balanced order over repeated measures. All repeated sessions were matched within subjects for time of day, day of week, and fixed dose treatments. Half of each drug subgroup was tested for 2 sessions between 6-10 a.m.; the other half was tested between 6-10 p.m. for both sessions. Pentobarbital-induced narcosis was timed as the duration marked by loss-regain of the righting reflex as defined in Experiment 2. Animals from different drug treatment subgroups were randomly assigned to different session periods, daily injection times, and order of interinjection durations (short followed by long vs. long followed by short). There were 12 experimental sessions yielding 120 dependent variable observations.

Experiment 4--Pentobarbital Assay 1

Purpose

Biochemical assays of mouse brain levels of pentobarbital were obtained following TRH-plus-barbiturate vs. saline-plus-barbiturate treatments. This procedure was undertaken to determine the peptide effect upon barbiturate

level. If a peptide-induced alteration of drug level was found, this would imply that the TRH analeptic effect is partially a function of such alterations in barbiturate disposition. This result would entail modification of the analeptic receptor hypothesis. On the other hand, if TRH were not to affect barbiturate concentration, the receptor hypothesis would be strengthened.

Subjects

A total of 45 male CF-1_S mice, four months of age and weighing 35-52g, were used. These mice were previously subjected to various TRH-pentobarbital treatments in an earlier experiment (Experiment 3). Therefore, these mice were not subjected to further drug treatments for a one month period prior to the present experiment in order to obviate unknown drug effects, e.g., receptor sensitization effects. The general housing and feeding conditions for these animals were the same as in previous studies (Experiments 1, 2, and 3). At the start of Experiment 4, animals were randomly reassigned from all prior treatment subgroups to each new block of treatments. The only restriction on random assignments was the requirement that new blocks be relatively equally matched in their heterogeneity, i.e., equal representation of previously treated subjects. The reasons for this latter assignment procedure were: (a) to effect greater homogeneity of unknown subject variabilities, i.e., carryover effects from prior drug treatments across all new subgroups ("homogeneity of variance" requirement); (b) to

approximate gaussian-like distributions of subjects within subgroups ("normality" requirement). These reasons seemed especially important in light of the relatively small cell sizes utilized.

Materials and Apparatus

Pharmacological agents. TRH was prepared as a 0.4% (W/V) solution in physiological (0.9% NaCl) saline vehicle for central (i.vt.) administrations via a Hamilton microsyringe; sodium pentobarbital was prepared as a 0.5% (W/V) solution in saline vehicle for parenteral (i.p.) administration via a 1 ml tuberculin syringe. The drug preparations and syringe apparatuses have been described previously (Experiments 1 and 2).

Pentobarbital assay reagents. A 0.60 M sodium phosphate buffer solution, pH 5.5, was prepared as a mixture of 0.099 mole fraction of dibasic phosphate (Na_2HPO_4; J. T. Baker Chemical Co.) added to 0.901 mole fraction of monobasic phosphate (NaH_2PO_4; J. T. Baker Chemical Co.). This mixture was obtained by: (a) adding 33.12g of monobasic phosphate ($NaH_2PO_4 \cdot H_2O$; formula weight = 137.99) to 200 ml glass distilled deionized water to obtain a 1.2 M solution, (b) 6.43g of dibasic phosphate ($Na_2HPO_4 \cdot 7H_2O$; formula weight = 268.07) was added to 20.0 ml of glass distilled deionized water to obtain a 1.2 M mixture, (c) 180.2 ml of 1.2 M monobasic phosphate was added to 19.8 ml of 1.2 M dibasic phosphate to obtain a 200 ml phosphate mixture, (d) this mixture was diluted to 400 ml of 0.6 M phosphate buffer,

pH 5.5, by adding 200 ml glass distilled deionized water to the 200 ml phosphate mixture.

A 0.8 M sodium phosphate solution buffer, pH 11.0, was prepared by: (a) adding 10.20g of sodium hydroxide (NaOH, normal weight 40.00) to 25.5 ml of glass distilled deionized water to obtain 25.5 ml of 10 N sodium hydroxide solution, (b) adding 68.14g of anhydrous dibasic phosphate (Na_2HPO_4; formula weight = 141.96; J. T. Baker Chemical Co.) to 600 ml of glass distilled deionized water to obtain 600ml of a 0.8 M dibasic phosphate solution, (c) the total volumes of both hydroxide and dibasic phosphates were then thoroughly mixed to obtain 625.5 ml of a 0.80 M phosphate buffer, pH 11.0.

Reagent grade isoamyl alcohol was added to spectro-quality petroleum ether (Matheson, Coleman, and Bell Co.) in a mixture (V/V) ratio of 1.5%/98.5%. External pentobarbital assay standards of 10.0 µg/g tissue (St1), 15.0 µg/g (St2), and 20.0 µg/g (St3) were prepared from stock solutions and homogenates as follows: (a) 2.0 mg of sodium pentobarbital was added to 10.0 ml of glass distilled deionized water. Then, 1.0 ml of this solution was diluted with distilled water to a final volume of 10 ml of 20 µg/ml pentobarbital stock solution; (b) aliquots consisting of either 50 µl, 75 µl, and 100 µl of this stock solution were then added to 1.0 ml of 10% (W/V) untreated tissue homogenates to obtain St1, St2, and St3, respectively. An internal pentobarbital assay standard was obtained by adding 0.15 ml of pento-barbital stock solution to 3.0 ml of an unknown <u>in vivo</u>

pentobarbital treated 10% (W/V) tissue homogenate, which was then run through the assay procedure. This manipulation was equivalent to adding 10.0 µg pentobarbital/g tissue. 3.0 ml of 10% (W/V) tissue homogenate was prepared as a blank for the assay procedure. All sample, standard, and blank homogenates and reagents were kept on ice or refrigerated (0 °C) between use in the assay.

Procedures

General. A total of 45 mice were randomly assigned to treatment, standard, and "blank" groups as outlined in Table 3. Animals were treated sequentially in random order. The sodium pentobarbital level (50 mg/Kg i.p.) was a parameter for all treated mice. At 1 min. before receiving the barbiturate, 3 groups of animals (Groups I, V, VI) were injected (i.vt.) with saline vehicle; Group II received 20 µg TRH; and two other groups (III and IV) of mice received either saline vehicle or 20 µg TRH at 45 min. before pentobarbital. At 1 hour following barbiturate administrations, animals were killed by cervical dislocation and brains were rapidly removed and placed on ice and either saved as whole brains or dissected into appropriate regions as required. Tissues were wrapped in parafilm and placed in labelled test tubes which were stored in a freezer (-10 °C) until later assayed for pentobarbital. Group VI animals were killed at 2 hours after barbiturate injections. Groups VII and VIII did not receive any drug treatments, but were killed and whole brains removed and frozen.

Table 3

Random Treatment Assignments for Pentobarbital Assay

(4 Month Old Male Mice): Experiment 4

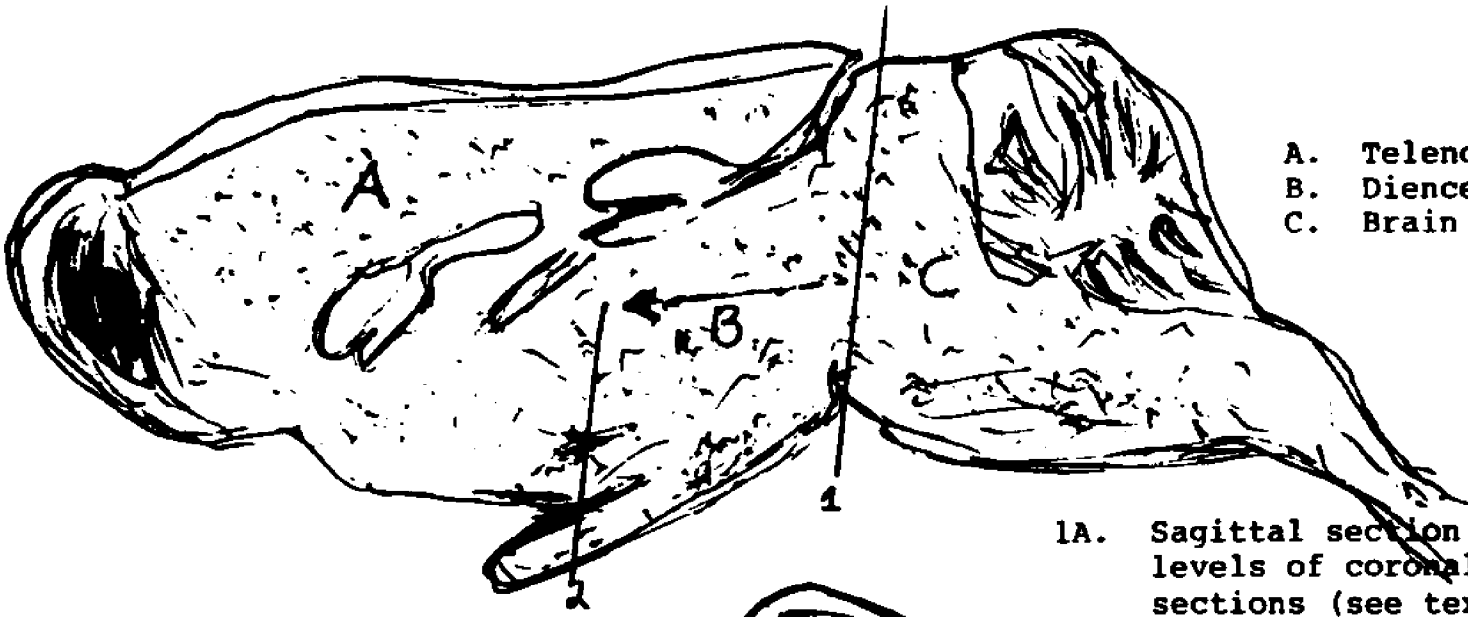
Central (i.vt.) Injections		Drug Administration Schedule	Schedule Killing and Brain Dissections
20 µg TRH	0.9% Saline		
Regional CNS tissues: Telecephalon, diencephalon, brain stem (n = 15)	Regional CNS tissues: Telencephalon, diencephalon, brain stem (n = 15)	TRH or Saline at 1 min. before 50 mg/Kg pentobarbital (i.p.)	At 1 hr. following pentobarbital injections
Whole Brains (n = 5)	Whole Brains (n = 5)	TRH or Saline at 45 min. before 50 mg/Kg pentobarbital (i.p.)	At 1 hr. following pentobarbital injections
-	Whole Brains (n = 2)	Saline at 1 min. before 50 mg/Kg pentobarbital	At 2 hr. following pentobarbital injections
-	"Internal Pentobarbital Standards"-Whole Brain (n = 1)	Saline at 1 min. before 50 mg/Kg pentobarbital	At 1 hr. following pentobarbital injections. Then, 10 µg/g pentobarbital standard were added. % recovery determined
-	"External Pentobarbibital Standards"-Whole Brain (n = 2)	None	Immediate kill, removal, and homogenization of tissues. Then 15-30 µ/g pent. standards were prepared from stock solution
-	"Blanks" Whole Brain (n = 2)	None	Immediate kill, removal, and homogenization of tissues

Dissection. Mice brains were dissected into three regions (Figure 1A) as follows (Sidman, Angevine, & Pierce, 1971; Slotnick & Leonard, 1975):

1. The lower "brain stem"--defined anatomically as a composite of the mes-, met-, and myelencephalon regions--was obtained by transsecting (Cut 1) dorso-ventrally with a scalpel through the superior colliculus-posterior commissure-cerebral aqueduct-pontine tegmentum, and then exiting anterior to the base of the pons and posterior to the mammillary bodies (Figure 1A).

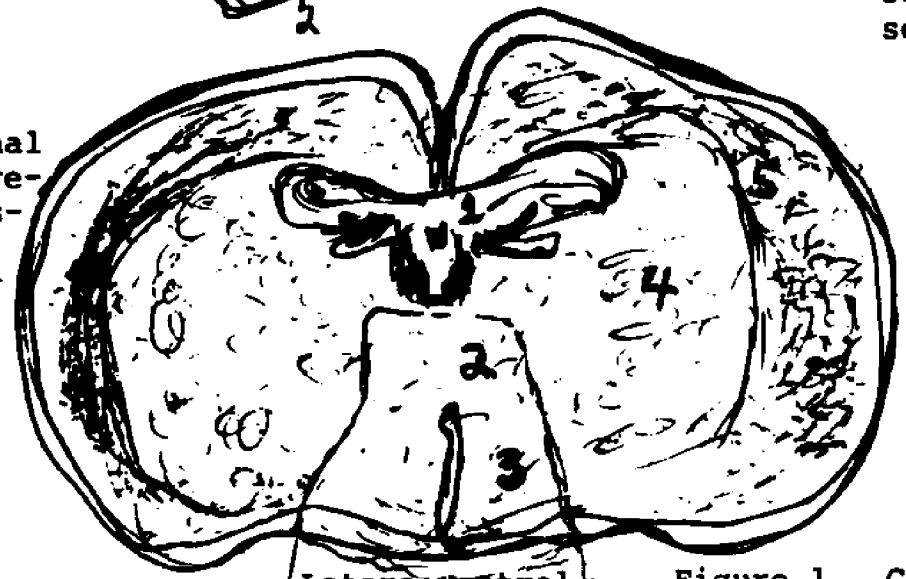
2. The "diencephalon" region consisted mainly of thalamus and hypothalamus. It was obtained by: (a) placing a ventro-dorsal coronal razor blade cut (Cut 2) from a point 1-1.5 mm anterior to the optic chiasm (anterior commissure level) and approximately 3.5-4.0 mm deep (Figure 1), (b) placing a scalpel 2.5-3 mm anteriorly into the caudal-most aspect (mammillary body level) of the remaining fore-brain section as far as the level of the anterior commissure, i.e., up to the point of the coronal razor cut (Figures 1A and B); placing the scalpel just beneath the hippocampus-septum system (approximately 3.5-4 mm dorsally) (Figure 1B), (c) placing two separate scoop-like latero-ventral cuts (Cuts 3 and 4) approximately $\pm$ 2-2.5 mm lateral with the scalpel, (d) scooping out the diencephalon tissue with a flat spatula (Figure 1B).

3. The "telencephalon" region--consisting of cerebral cortex, olfactory bulbs, corpus callosum, hippocampus, septum,



A. Telencephalon
B. Diencephalon
C. Brain stem

1A. Sagittal section indicating levels of coronal transsections (see text).



1B. Coronal Section of Forebrain to Illustrate Latero-ventral Transsections Procedure to Attain Isolation of the Diencephalon.

1. Hippocampal complex
2. Thalamus
3. Hypothalamus
4. Caudate N./putamen
5. Neocortex

Figure 1. Coronal Transsections.

fornix, neostriatum--was the remaining forebrain tissue.

Pentobarbital assay. The pentobarbital assay was modified from the methods of Brodie, Burns, Mark, Lief, Bernstein, and Papper (1953) and Richter (1979). Three samples were pooled for each region represented. Telencephalon, brain stem, and whole brain tissues were prepared as 10% (W/V) homogenates. Standards and blanks were also prepared from these homogenates. Diencephalon tissues were prepared as 5% homogenates. As mentioned, all reagents were kept on ice or refrigerated (0 °C) between use. 3.0 ml of the homogenates were then transferred to 40 ml capacity glass (Kimax) centrifuge tubes which also contained 0.5g of sodium chloride. 30 ml of spectroquality petroleum ether which contained 1.5% isoamyl alcohol was added. The solutions were shaken in a horizontal position at 200 cycles/min. for 45 min. on a Burrell wrist-action shaker. They were then centrifuged at 1500 revolutions per minute (r.p.m.) for 3 min. in a GLC-1 centrifuge to separate the layers. The barbiturate-containing organic (ether) upper layer was aspirated, via a hand-held aspirator mounted on a 10 ml pipette, and then saved. The bottom aqueous layer was discarded. The organic phase was then transferred to a new centrifuge tube which also contained 4.0 ml cold 0.80 M alkaline (pH 11.0) phosphate buffer. The tubes were shaken on the Burrell shaker at 200 cycles/min. for 3 min., and then centrifuged at 1500 r.p.m. for 3 min. to separate the layers. The top organic (ether) layer was aspirated and discarded.

2.0 ml of the bottom pentobarbital-containing aqueous alkaline (pH 11.0) phase was transferred to a quartz cuvette. Absorption values of samples were then read in an ultraviolet (Beckman DB-G grating) spectrophotometer at a wavelength (λ) setting of 240 nm. Blank values were subtracted from unknown samples and standards to obtain corrected values. Corrected sample values were then compared to corrected internal and external standards to determine regional and whole brain pentobarbital concentrations as well as the efficiency (% recovery) of the procedure.

Experiment 5--Behavioral Pharmacology 3

Purpose

The results obtained from the previous pharmacological studies (Experiments 2 and 3) indicated that the animal's age was a relevant factor in the TRH analeptic effect. In those studies, TRH was injected (i.vt.) at 1 min. before pentobarbital injections. Therefore, the present study was designed to further investigate the age factor, as well as to determine whether the peptide was also effective when centrally infused after barbiturate administrations.

Subjects

There were two different matched age groups of male CF-1_S mice in this study: two months vs. three months. These ages were chosen because they matched the ages of mice in the earlier TRH pharmacology studies where significant age effects had been found (Experiments 2 and 3). General handling and housing environments of the animals in the

present study were similarly matched to conditions of the earlier studies. Since there was only a one month age difference between the two groups, they were relatively closely matched in body weights (B.W.). The three month olds were only slightly heavier than the two month olds: The former group had a mean B.W. (±S) of 35 ± 3g, while the latter's was 32 ± 3g.

Materials

The same materials were used in this study as in the earlier pharmacology studies: 0.4% TRH solutions; 0.5% pentobarbital solutions; physiological saline vehicles; microsyringe instruments; tuberculin syringes and needles; and testing cages.

Procedure

Twenty mice from each age group were randomly assigned to treatment conditions as displayed in Table 4.

Table 4

Treatment Assignments: Experiment 5

	Saline		vs.	20 µg TRH	
	1 min.	5 min.		1 min.	5 min.
2 months	(n=5)	(n=5)		(n=5)	(n=5)
vs.					
3 months	(n=5)	(n=5)		(n=5)	(n=5)

Note. 50 mg pentobarbital/Kg B.W. was a parameter.

The pharmacological experiment was performed over two successive evening sessions (beginning at 5 p.m.) which lasted 5.5-6.5 hrs. each. All two month old mice were tested during the first session; all three month olds were tested during the second session. The two sessions were equally matched for order of treatment conditions. The order of testing within sessions was: TRH groups were studied prior to saline control groups; groups receiving central administration at 1 min. after barbiturate were tested prior to groups receiving administrations at 5 min. after barbiturate. Animals within given treatment subgroups were tested in random order. No attempt was made to counterbalance subconditions or age within sessions because: (a) treatment subconditions were brief, taking only 0.5-0.75 hr. to complete the administrations, (b) diurnal variation factors were found to be unimportant as revealed by Experiment 3 (see Results), (c) counterbalancing the age factors within sessions would entail that the more subtle temporal factors would no longer be balanced, (d) within-group (cell) homogeneity would be destroyed by the accrual of nonspecific experimental error variability which accompanies rapidly shifting technical procedures.

The dependent variable was pentobarbital-induced narcosis, which was measured by the righting reflex as defined in the earlier pharmacology experiments.

Experiment 6--Pentobarbital Assay 2

Purpose

Results from behavioral pharmacology studies with mice (Experiments 2, 3, and 5) indicate that profound changes in drug sensitivities to TRH-pentobarbital interactions occur within the narrow postnatal development stage of 2-3 months. The earlier studies indicated that the peptide was an effective analeptic whether centrally administered (i.vt.) before or after barbiturate injections. In addition, the previous results also indicated that central infusions of TRH before barbiturate inductions do not affect barbiturate metabolism in four month old mice. The present experiment was performed to discern whether the age-related pharmacological effects could be explained by differences in regional brain levels of pentobarbital. In this biochemical study, TRH was administered to mice at 5 min. after barbiturate-induced narcosis (loss of the righting reflex). The barbiturate assay was performed on animals that had been previously tested under these same experimental conditions for behavioral pharmacology activities (Experiment 5).

Subjects

Two-month-old and three-month-old all males CF-1_S mice tested 1 week earlier in pharmacology studies (Experiment 5) were again subjected to similar drug treatments in the present study for subsequent biochemical assays of brain barbiturate levels. The general handling and housing conditions of these animals were already described in the earlier

study.

Materials and Equipment

Pharmacological agents. Aliquots of 0.4% TRH solutions in saline vehicle were prepared for later 20 µg/5 µl central (i.vt.) administrations via the microsyringe instruments. Sodium pentobarbital was prepared as a 0.5% solution in saline vehicle for later parenteral (i.p.) injections via the tuberculin syringe instruments. Physiological saline was used for central and parenteral control injections where required.

Pentobarbital assay reagents and instruments. Acid phosphate buffer was prepared by dissolving 27.0g of monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$; formula weight = 137.99; J. T. Baker Chemical Co.) and 1.00g of anhydrous dibasic sodium phosphate (Na_2HPO_4; formula weight = 141.96; J. T. Baker Chemical Co.) in 1.0 L of cold glass-distilled deionized water. A 0.2 M solution of alkaline phosphate buffer, pH 12.0, was prepared by dissolving 72.0g of trisodium phosphate ($Na_3PO_4 \cdot 12H_2O$; formula weight = 380.18; J. T. Baker Chemical Co.) in 1.0 L of cold glass-distilled deionized water which was then stored in a polyethylene bottle. The phosphate buffers were kept refrigerated (0 °C) between use. The chloroform ($CHCl_3$; formula weight = 119.38) used for barbiturate extractions was a spectroquality reagent (SpectrAR; Mallinckrodt).

External pentobarbital assay standards of 10.0 µg/g, 15.0 µg/g, and 20.0 µg/g tissue, an internal standard

(equivalent to 10.0 μg pentobarbital added/g), and tissue blanks were prepared as described in Experiment 4. Fluorescence of the extracted barbiturate samples, standards, and blanks were read in a ratio fluorometer (Farrand Optical Co., Inc.). The excitation (primary) filter was a Corning glass filter #7-54 which displays 40% transmittance at a λ of 260 nm and 60-70% at 276 nm; the fluorescence (secondary) filter was a Corning glass filter #3-73 which displays 50-60% transmittance at a λ of 430-440 nm (personal communication with Steven Mahdesian, Manager, Farrand Optical Co., Inc.). These filter characteristics delineate the fluorescence profile of pentobarbital in alkaline solutions (Gifford, Hayes, King, Miller, & Burns, 1974; Hollister, Kanter, & Clyde, 1963).

General Experimental Procedure

Twenty mice from each age group--two months vs. three months--were assigned to the same drug treatments for the barbiturate assay as they had been in the behavioral pharmacology studies (Table 5). The only difference in procedure between the two studies was the fact that in the present study TRH and/or saline was administered only during post-narcosis (loss of righting reflex), but not during the pre-narcosis periods.

Starting at 3 p.m. and working through the evening, mice from each group were treated sequentially in random order over 2 successive sessions. Sodium pentobarbital was administered (i.p.) first at a parameter dose of 50 mg/Kg.

Table 5

Treatment Assignments: Experiment 6

	Saline Control	vs.	20 µg TRH
2 months	5 min. post-narcosis (n = 10)		5 min. post-narcosis (n = 10)
3 months	Same procedure (n = 10)		Same procedure (n = 10)

Note. 50 mg pentobarbital/Kg B.W. was a parameter.

At 5 min. after initial narcosis (failure to right 3 times/min.), animals were given central (i.vt.) injections of either 20.0 µg TRH or saline in 5 µl physiological saline vehicle. Then, at 1.5 hr. after initial narcosis, animals were killed by cervical dislocation followed by removal and dissection of brains over ice (0 °C). The specific details of the dissection procedure have already been described (Experiment 4), and a general outline of tissue pooling for the biochemical assay is provided in Table 6. The three CNS regions were the telencephalon (consisting of the cerebral cortex, olfactory bulbs, corpus callosum, hippocampus, septum, fornix, and neostriatum), the diencephalon (thalamus and hypothalamus), and brain stem (including mes-, met-, and myelencephalon). Regional tissues were wrapped in parafilm and stored in a freezer (-10 °C) until later assayed.

Table 6

Outline of Brain Tissue Collection

		Saline[a] Control	vs.	20 µg TRH[a]
2 months[b]	Tel[c]:	3 pooled sample assays (Σ10 tissues/CNS region)		3 pooled sample assays (Σ10 tissues/CNS region)
	Dienc[d]:	Same procedure		Same procedure
	B.S.[e]:	Same procedure (from 10 dissected mice brains)		Same procedure (from 10 dissected mice brains)
3 months[b]	Tel:) Dienc:) B.S.:)	Same procedure as for 2 months (from 10 dissected mice brains)		Same procedure as for 2 months (from 10 dissected mice brains)

[a]Administered (i.vt.) at 5 min. post-narcosis induced by 50 mg pentobarbital/Kg B.W. as a parameter.

[b]Standards and blanks were also prepared and run through the assay procedure.

[c]Tel = telencephalon

[d]Dienc = diencephalon

[e]B.S. = brain stem

Pentobarbital assay. The pentobarbital assay was modified from Hollister et al. (1963). All tissue samples, standards, and blanks were prepared as a 5% (W/V) homogenate in glass-distilled deionized water. 5.0 ml aliquots of samples were transferred to 40 ml glass, round bottom (Kimax) centrifuge tubes. One milliliter of the acid phosphate buffer was added, followed by 10.0 ml of the spectroquality chloroform. The tubes were shaken in a horizontal position for 20 min. at 200 cycles/min. on a Burrell wrist-action shaker. They were then centrifuged at 800g (relative centrifuge force; RCF) for 5 min. in a GLC-1 centrifuge to separate the 2 phases. The aqueous (upper) layer was then aspirated and discarded. A 5.0 ml aliquot of the organic (lower) phase was then transferred to a clean 40 ml capacity glass, round bottom (Kimax) centrifuge tube. 10.0 ml of the alkaline phosphate buffer was added, and the tubes were then shaken for 5 min. The 2 phases were then separated by again centrifuging at 800g for 5 min.; 3.0 ml of the alkaline aqueous barbiturate extract were removed as the upper phase and transferred to quartz cuvettes. Barbiturate fluorescence was then read in a ratio fluorometer (Farrand Optical Co., Inc.) set at a slit opening of "1" (minimal setting) with an excitation λ of 260-276 nm (Corning glass filter #7-54 as primary) and a fluorescence λ of 430-440 nm (Corning glass filter #3-73). Blank fluorescence readings were subtracted from unknown sample and standard values. The corrected sample values were then compared to standards

to calculate regional brain barbiturate concentrations.

Experiment 7--Radioreceptor Assays

Purpose

Several important findings implicate TRH as a neuromodulator: TRH has widespread and extremely high concentrations in the brain; it is localized within nerve axon terminals; it has marked modulatory influences upon neuronal activities (altered discharge frequencies, transmitter release, and transmitter turnover); it has profound pharmacological actions; and it has been further demonstrated that there are high concentrations of specific high-affinity TRH binding sites distributed in the CNS. Thus, the present experiment was designed to test the hypothesis that the TRH analeptic mechanism is mediated at these same specific central binding sites. In vitro competitive displacement [^{3}H]-TRH radioreceptor assays were performed to assess antagonistic relationships between TRH and barbiturate analogues at central TRH receptors. This radioreceptor study has utilized purified presynaptic vs. postsynaptic receptor membrane preparations (synaptosomal fractions) obtained from different regional mouse brain tissues in order to further localize these activities.

Subjects

Fifty male CF-1$_S$ mice (Charles River Breeding Labs), weighing 32-41g and three months of age, were used. The mice were maintained on Purina Chow and tap water ad libitum, and were housed 3 animals per cage in quarters kept at

constant room temperature (23-25 °C) with alternating 12 hours light/12 hours dark periods.

Materials and Equipment

Synaptic membranes. The reagents utilized for the receptor preparations included: 0.32 M, 0.8 M, and 1.2 M sucrose (molecular weight = 342.3) solutions prepared in glass-distilled deionized water; a pH 7.4 buffer (Buffer A) prepared as a mixture of 0.25 M sucrose, 5 nM tris-HCl (molecular weight = 158.0), and 1 mM magnesium chloride ($MgCl_2 \cdot 6H_2O$, formula weight = 203.3) in distilled water; a pH 7.5 buffer (Buffer B) was prepared as a mixture of equal parts of 50 mM tris-HCl and 10 mM magnesium chloride; and a pH 7.5 buffer (Buffer C) was prepared by mixing equal parts of 75 mM tris-HCl and 25 mM magnesium chloride. Materials utilized included polyallomer and cellulose nitrate tubes, plastic disposable pipettes, and a pipette gun (Clay Adams). The equipment utilized for receptor preparations included: a make-shift homogenizer consisting of a teflon rotor mounted on an electric drill (Black & Decker Model #30-Type E) which was clamped vertically downwards on a ringstand (thus allowing a glass homogenizer to be placed underneath); a refrigerated superspeed centrifuge (Sorvall Model #RC-B) equipped with either a large rotor (Model #HG-4, 9.83 in. radius) or a small rotor (Model #SS-34, 4.25 in. radius); and a freezer-containing ultracentrifuge (Beckman Model #L2-65B) with a swingbucket rotor (Model #SW-27, 7.25 in. radius).

Protein assay. The reagents utilized to assay protein

content of receptor membranes included: a freshly prepared reagent mixture (Reagent A) which consisted of 1 ml of 1% sodium tartrate, 1 ml of 5% cupric sulfate ($CuSO_4 \cdot 5H_2O$, molecular weight = 249.68), and 50 ml of 2% sodium carbonate (Na_2CO_3, molecular weight = 93.98); a phenol reagent was prepared by mixing 1 ml of phenol with 2.5 ml glass-distilled deionized water; and bovine serum albumin (BSA) standards--15.0 µg/ml (BSA1), 20.0 µg/ml (BSA2), 25.0 µg/ml (BSA3), and 30.9 µg/ml (BSA4)--were prepared by adding 150-300 µl/aliquots of a 0.01% (W/V) BSA stock solution to 0.4 ml of 1 N sodium hydroxide (NaOH, normal weight = 40.00) and 0.5 ml of glass-distilled deionized water; and blanks were prepared from 0.5 ml of 1 N sodium hydroxide added to 0.5 ml of the distilled water. A spectrophotometer (Spectronic 20 Model, Bausch-Lomb) was utilized to detect absorption of the chromagen for protein determinations.

Radioreceptor assay. The reagents utilized for the receptor assay included: Thawed aliquots of 5 nM [^{3}H]-TRH (molecular weight = 362.4, specific activity = 115.0 Ci/mmol, New England Nuclear) which possessed a 99% level of radiochemical purity (determined by chromatographic assays) were used for the radioligand; thawed aliquots of 10 µM, 25 µM, and 50 µM TRH (molecular weight = 362.4, Sigma Chemical Company) were used for the competing ligands; a 50 µM sodium pentobarbital (molecular weight= 248.26) solution, freshly prepared as needed by adding 1.24 mg of the barbiturate to 100 ml of glass-distilled deionized

water, was utilized as a competitive inhibitor in the radioreceptor assay; a second 50 μM barbiturate competitive inhibitor was freshly prepared by adding 1.27 mg of sodium phenobarbital (molecular weight = 254.22) to 100 ml of the distilled water; a third 50 μM inhibitor solution was freshly prepared by adding sodium thiopental (molecular weight = 264.33) at 1.32 mg/100 ml water; and a special pre-mixed solvent- and solubilizer-containing primary fluor scintillation cocktail (Biofluor "High Efficiency Emulsifier Cocktail", New England Nuclear) was later added to the assay reagents to develop the scintillation counting efficiency. Equipment and supplies utilized during the receptor assay included: an incubator (Dubnoff Metabolic Shaking Incubator); 15 ml capacity filtration funnels (Millipore Model #XX10-025-14) which held glass fiber filters (Whatman Type GF/B, 2.4 cm diameter) were clamped (Clamp Model #XX10-025-3, Millipore Corporation) to a specially designed 6-well manifold-type filter machine; forceps and latex surgical gloves; and a liquid scintillation counter (Model # Delta 300, Searle Analytic Inc.).

Procedure

General. A total of 50 mice were randomly selected, with the single restriction of equal representation from all previously treated groups. Animals were successively killed by cervical dislocations, brains were rapidly removed, placed on glass plates over ice, and then dissected into several brain regions. These tissues were placed into

appropriately labelled test tubes. Regional brain tissues were obtained for presynaptic and postsynaptic receptor membrane preparations during two separate sessions. Once all tissues were obtained during a given session, respective membrane fractions were rapidly prepared.

Dissection. Mouse brains were dissected into 3 regions as follows (Figure 2) (Sidman et al., 1971; Slotnick & Leonard, 1975):

1. The cerebral cortex was obtained by placing a scalpel immediately anterior to the superior colliculus, and then making a horizontally-oriented anterior arching transsection to circumscribe the dorsal aspects of the corpus callosum (from splenium to genu), and then exiting slightly ventrally at the anterior olfactory nucleus;

2. The "limbic forebrain"--consisting of the hippocampus, septum, fornix, neostriatum, hypothalamus, and thalamus--was obtained by a ventral coronal transsection from the superior colliculus to the posterior aspect of the mammillary bodies;

3. The "brain stem"--anatomically defined as the mes-, met-, and myelencephalon--was the remaining caudal brain tissue.

Presynaptic receptor membranes. Purified presynaptic membrane subcellular fractions were obtained by differential and density gradient centrifugation procedures (modified from Essman, W. B., & Essman, S. G., 1977; Israel & Whittaker, 1965; Whittaker & Barker, 1972). Regional brain tissues

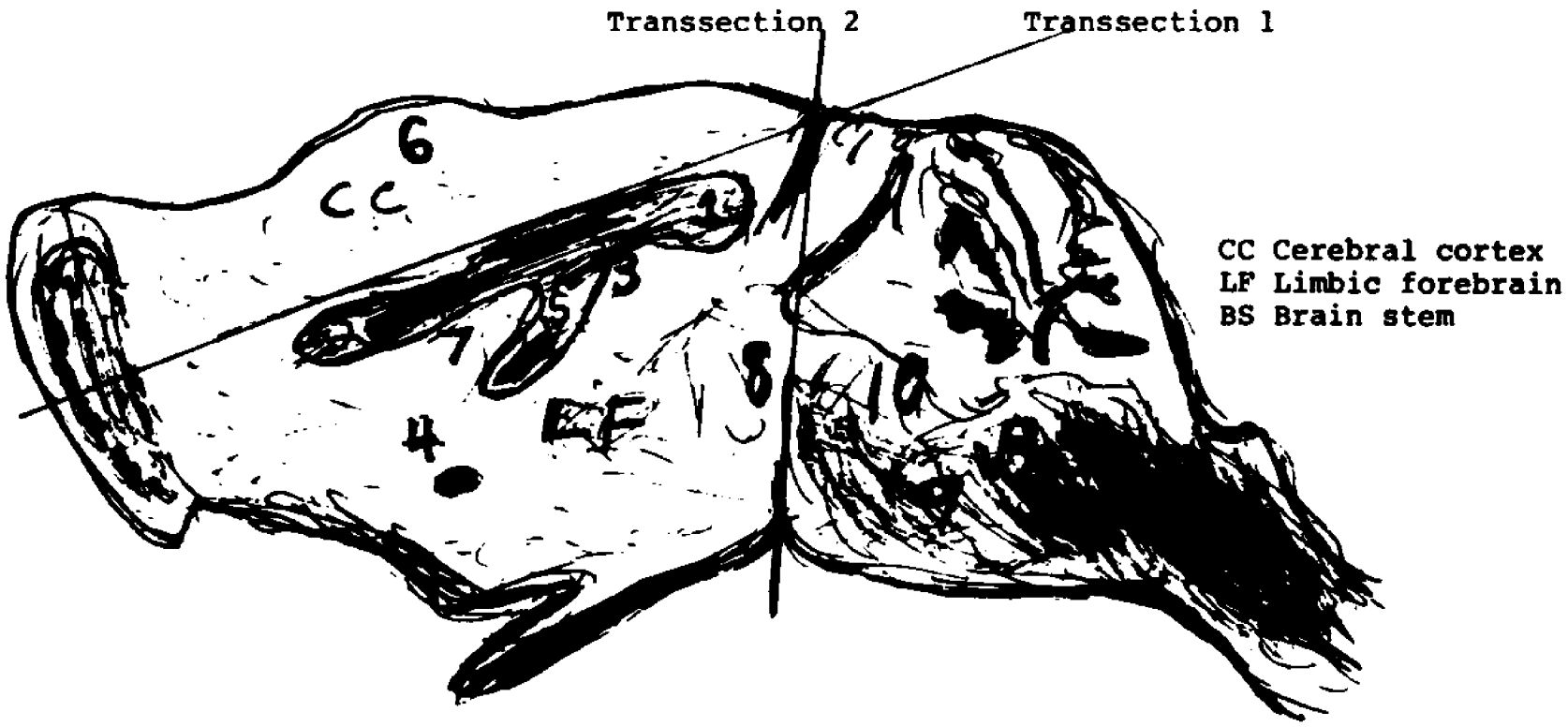


Figure 2. Sagittal Section Indicating Orientations of Transsections to Isolate Cerebral Cortex, Limbic Forebrain, and Brain Stem.

from 25 mice were pooled as 12-13 tissues/homogenate prepared as a 10% (W/V) suspension in ice cold 0.32 M sucrose via the teflon-and-glass homogenizer (with a rotation rate of 2000 r.p.m.) using 10-15 strokes/homogenate preparation. Aliquots of the suspension were transferred to glass tubes and placed into the refrigerated Sorvall superspeed centrifuge (set at 5 °C) equipped with the HG-4 (large) rotor. The suspension was then centrifuged at 2500g (3000 r.p.m.) for 10 min. The crude mitochondrial-containing fraction supernatant (crude S1) was then removed and saved in plastic tubes. 4.0 ml of ice cold 0.32 M sucrose was added to the pellet (P1). The resuspended P1 was then centrifuged (5 °C) at 2500g (3000 r.p.m.) for 10 min. A purified residual supernatant (purified S1) was thus obtained and was then pooled with the crude S1. The high protein-containing nuclear fraction, which had sedimented to the bottom of the tube, was discarded. The combined S1 was then transferred to polyallomer tubes, and then centrifuged (5 °C) at 17,300g (12,000 r.p.m.) for 1 hr. in the Sorvall centrifuge equipped with the SS-34 (small) rotor. The obtained supernatant (S2) was then discarded. The mitochondrial fraction-containing pellet (P2) was resuspended with 2.0 ml of 0.32 M sucrose. 7.5 ml of 1.2 M sucrose was then poured into 17 ml cellulose nitrate tubes. 7.5 ml of 0.8 M sucrose was then layered on top. Then the resuspended P2 (in 0.32 M sucrose) was carefully layered on top. This was effected by slowly rotating the cellulose tube, which was held in an angled position,

during the very slow delivery (<0.1 ml/30 sec.) of suspension via disposable pipettes mounted on a Clay Adams pipette gun. The cellulose tubes containing this layered density gradient suspension were gently hooked vertically into the SW-27 swingbucket rotor which was then mounted into the Beckman ultracentrifuge. The centrifuge freezer was set at 5 °C and > 1 atm. The suspension was then centrifuged at 78,000g (25,000 r.p.m.) for 1.25 hr. This procedure thereby produced 3 separate subcellular fractions suspended across the sucrose density gradient. A highly purified synaptosomal fraction was found to be isolated at the 0.8 M and 1.2 M sucrose-fraction interface (middle B Band layer). Small myelin fragments were suspended above the 0.8 M fraction (upper A Band layer), and an intraterminal mitochondrial fraction was recovered in the sedimented pellet below the 1.2 M sucrose (lower C Band layer). The separate fractions were carefully removed via aspiration using glass disposable micropipettes and the pipette gun. The purified presynaptic receptor membranes were then lysed by hypo-osmotic shock in glass-distilled deionized water, and stored in a freezer (-10 °C) until used in the radioreceptor assay. Separate aliquots of the receptor membranes were subjected to a protein determination assay.

Postsynaptic receptor membranes. The following differential and density gradient subcellular fractionation procedure developed by Essman, E. J. and Essman, W. B., 1980 (as modified from Cotman & Taylor, 1972; and Cotman,

Banker, Churchill, & Taylor, 1974) yields highly purified postsynaptic receptor membranes. Thawed regional mouse brain tissues from 25 adult male CF-1_S mice were pooled at 12-13 tissues/homogenate prepared as a 10% (W/V) suspension (S Buffer) in ice cold sucrose-magnesium chloride, pH 7.4 Buffer A (see Materials--synaptic membranes). This was effected by utilizing the teflon-and-glass homogenizer as previously described. The S Buffer was then centrifuged at 1000g (2800 r.p.m.) for 10 min. in the Sorvall centrifuge (5 °C setting) mounted with the SS-34 rotor. The obtained supernatants (S1) were pooled 2/regional tissue and then spun at 40,000g (18,000 r.p.m.) for 10 min. in the Sorvall centrifuge. The pellets were next washed in the pH 7.5 Buffer B, and then membrane preparations were resuspended in the pH 7.5 Buffer C. Postsynaptic membrane preparations were stored in a freezer (-10 °C) until utilized. Aliquots of the membranes were assayed for protein concentration levels.

Protein determinations. Both the presynaptic and postsynaptic membrane fractions derived from regional mouse brain tissues were assayed for protein concentration levels by a modified Folin-Phenol Method (Lowry, Rosebrough, Farr, & Randall, 1951). 0.5 ml aliquots of membrane fractions were transferred to test tubes. 0.5 ml aliquots of glass-distilled deionized water were added. The solution was allowed to stand for a few minutes to check for turbidity. 5.0 ml of freshly prepared reagent A (see Materials) was

then added. The solution was mixed thoroughly on a Vortex mixer and allowed to stand for 17 min. at room temperature. 0.2 ml of phenol reagent was then added and mixed immediately. The mixture was allowed to stand for 20 min. The absorption of the chromagen was read in a Spectronic 20 spectrophotometer at 660 nm λ.

Radioreceptor assay. The competitive displacement radioreceptor assays utilized [^{3}H]-TRH as a radioligand, TRH as a competing ligand, and three barbiturate analogues as competitive inhibitors. Presynaptic vs. postsynaptic receptor assays were run during separate sessions, although comparable procedures were utilized. Within sessions, assays were performed on receptors derived from three separate mouse brain areas (see Dissection): the cerebral cortex, the limbic forebrain, and the brain stem. Latex surgical gloves and stainless steel forceps were used when necessary to handle radioactive contaminated materials. All synaptosomal and TRH solutions were in the same corrected pH buffers as utilized for membrane preparations. 0.1 ml aliquots of duplicate synaptic receptor membranes were transferred to disposable plastic test tubes. 1.0 ml aliquots of 10 μM, 25 μM, and 50 μM TRH vs. buffer were added to various incubations of presynaptic receptors, and 50 μM TRH vs. buffer aliquots were added to postsynaptic receptors. 1.0 ml aliquots of 50 μM barbiturate analogues--including the sodium salts of pentobarbital, phenobarbital, and thiopental--were added to appropriate incubations as required

and compared to buffer additions. 0.1 ml of 5 nM [^{3}H]-TRH was then added to the sample mixtures. Competitive binding reactions were then allowed to incubate at 37 °C for 1 hr. in a Dubnoff Metabolic Shaking Incubator. At the end of the incubation period, membrane-bound radioligands were separated from free radioligands by three repeated rapid washings of samples with cold wash buffer over Whatman CF/B glass fiber filters. The filters were then placed in filtration funnels connected to a manifold vacuum filtration system. After washing, the sample-containing fiber filters were transferred to scintillation vials, and 10 ml of biofluor scintillation cocktail was added per sample. Bound radioactivity was then determined by reading the sample vials in a Searle Delta 300 Liquid Scintillation Counter at an efficiency of 98%. Count rates were averaged over 50 min. per sample. Specific binding was defined as the amount of radioligand displaced from the receptor by an excess ($10^4 K_D$) of unlabelled ligand, i.e., the difference between total and non-saturable binding. Specific binding (Bmax), dissociation constant (K_D), and competitive inhibition values in the radioreceptor assay were determined for each CNS region and for each membrane receptor type by the procedures outlined by a number of workers (Akera & Cheng, 1977; Cheng & Prusoff, 1973; Jacobs, Chang, & Cuatrecasas, 1975; Levitzki, Sevilia, Atlas, & Steer, 1975; Rodbard, Bridson, & Rayford, 1969; Rodbard & Frazier, 1975; Rubinow, 1977; Segal, 1975; Smith, 1977; Thorell & Larson, 1978). Additional dissociation

radioreceptor assays were performed to observe the kinetics of TRH activity at its specific receptor sites. These assays utilized the same procedures discussed above, except that receptors were pre-incubated for 1 hour with radioligand alone, which was then followed by infinite dilutions via additions of excess unlabelled ligand.

Experiment 8--Radiochemical Assays

Purpose

The previous experiment (Experiment 7) tested the viability of the hypothesis that the TRH analeptic mechanism is mediated at specific central TRH binding sites. Radioreceptor assays were performed to assess the degree of specificity of TRH-barbiturate interactions within these sites. The assays also afforded the additional discrimination between presynaptic vs. postsynaptic receptors at different levels of the mouse brain. The present *in vivo* radiochemical assays were undertaken to further validate the earlier *in vitro* findings. Live mice received tritiated TRH i.vt. to investigate whether the peptide would distribute to the same brain sites as displayed in the receptor binding assays. The study was also undertaken to investigate whether parenterally administered pentobarbital would displace the peptide from the same brain sites as found in the binding study.

Subjects

Six male CF-1_S mice, weighing 33-39g and three months of age, were used. These mice were cared for in the same manner as described for previously tested animals.

Materials and Equipment

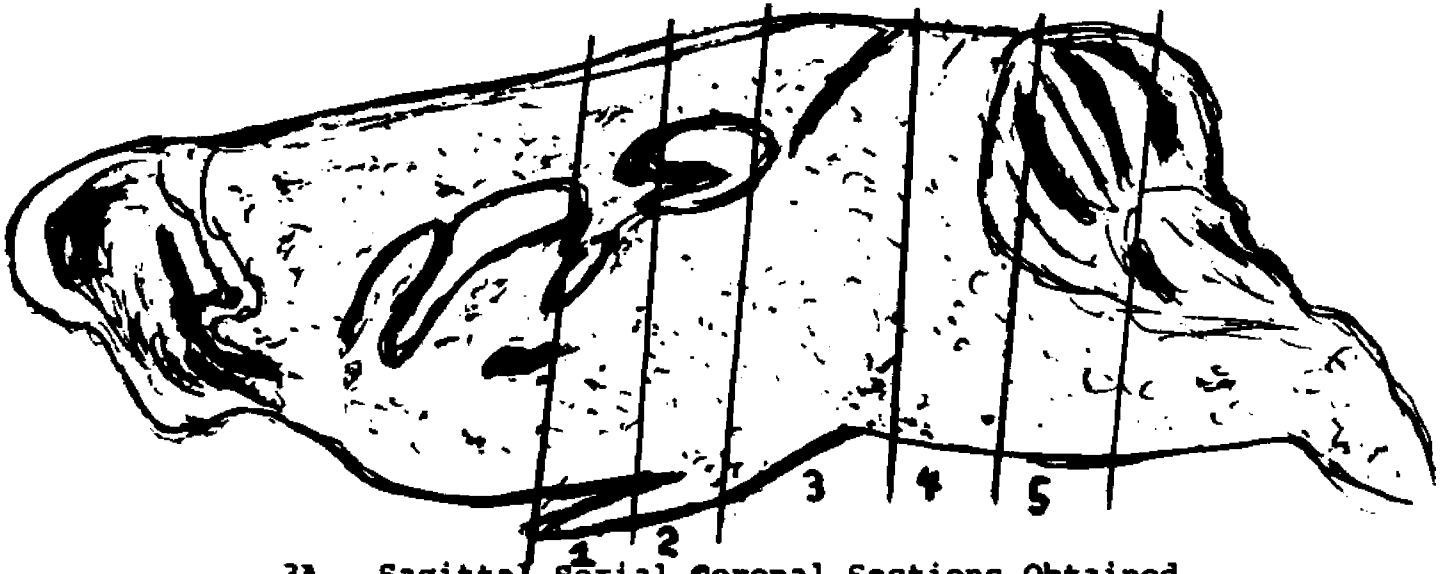
Thawed aliquots of 5 nM [^{3}H]-TRH (molecular weight = 362.4, specific activity = 115.0 Ci/mmol, New England Nuclear) were used for i.vt. administrations via the microsyringe apparatus described earlier (Experiment 1). Sodium pentobarbital (molecular weight = 248.26) was prepared as a 0.5% (W/V) solution for parenteral (i.p.) injections via the tuberculin syringe. Assay reagents utilized included glass-distilled deionized water, Triton X-100 solubilizer detergent (Rohm and Haas Co.) and Biofluor liquid scintillation cocktail (New England Nuclear). Equipment included a Supermixer vortex mixer (Model #1290; Lab-Line Instruments), an Angle centrifuge (Ivan Sorvall Inc.), 15 ml capacity glass test tubes (Corex), and a Delta 300 liquid scintillation counter (Searle Analytic Inc.).

Procedure

General. Three mice from each of 2 groups successively received central (i.vt.) administrations of 25 fmol [^{3}H]-TRH /5 µl at 1 min. before parenteral (i.p.) injections of either physiological (0.9%) saline (Group 1) or sodium pentobarbital at 50 mg/Kg body weight (Group 2). It took 21 min. to complete the drug treatments. The order of treatments was randomized across all animals. The volume of parenteral injections was adjusted to 1% of the mouse's body weight. Animals were then killed by cervical dislocation at 1 hr. after barbiturate administrations. Whole mice brains were then rapidly removed and placed on glass plates over

ice (0 °C) as previously described. This procedure took 22 min. to complete.

Dissection. The whole brains were then successively dissected under a binocular microscope (Bausch-Lomb) at 1-10X ocular power. A thin (3.2 mm) transparent glass plate (rinsed with distilled water between dissections) was placed over the microscope dissecting stage and under the brain to prevent radioactive contamination of the microscope. Serial coronal sections of the brain, 1.0-2.5 mm thick, were then obtained with stainless steel razor blades and a scalpel (Figure 3A). These sections were made by placing coronal transsections: (a) 2 mm anterior to the optic chiasm and 1 mm anterior to the optic chiasm (section 1), (b) at the mammillary body (section 2), (c) anterior to the interpeduncular nucleus through the superior colliculus (section 3), (d) at the anterior cerebellum level (section 4), and (e) at the posterior cerebellum level (section 5). Each coronal section was dissected separately. Individual nuclei were obtained by circumscribing the specific area with disposable dissecting needles and the tip of a scalpel. The coronal sections and individual nuclei were identified by comparing the actual tissues viewed under the microscope against photomicrographs of these tissues presented in mouse brain atlases (Sidman et al., 1971; Slotnick & Leonard, 1975). In addition, several hand-made sketches of these tissues were drawn prior to the experimental session. As nuclei were obtained, they were placed into appropriately labelled glass test tubes placed on ice (0 °C). Tissues



3A. Sagittal Serial Coronal Sections Obtained

3B. Telencephalon Coronal: (a) Hippocampus, (b) Neostriatum, (c) Cerebral cortex

3C. Diencephalon Coronal: (a) Thalamus, (b) Hypothalamus

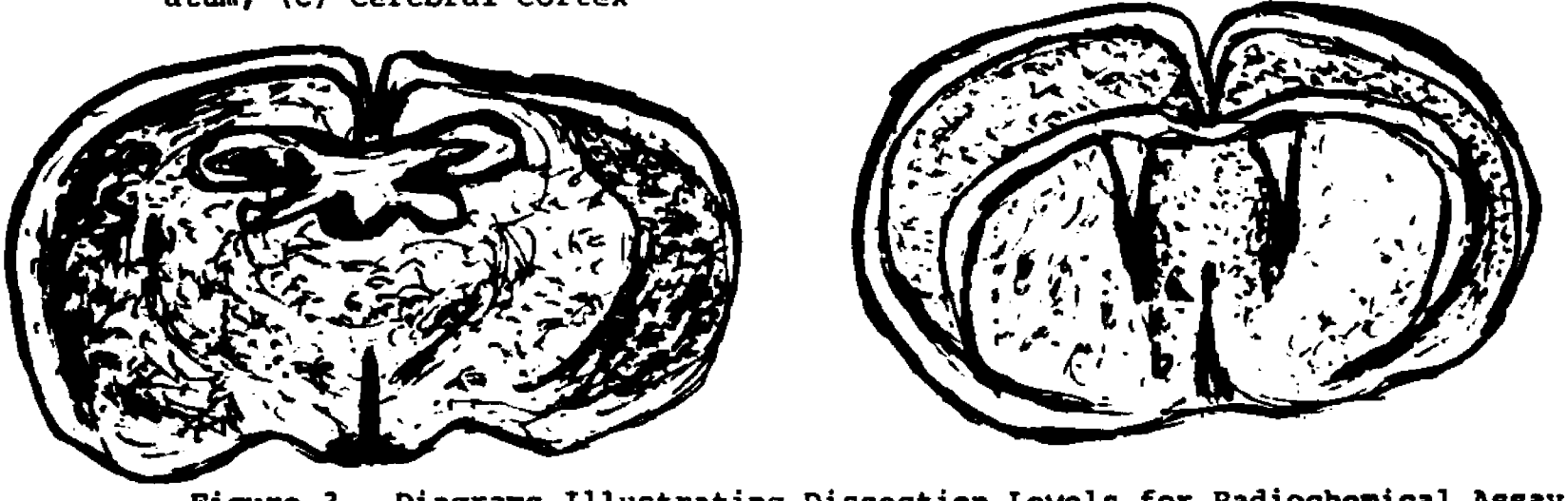


Figure 3. Diagrams Illustrating Dissection Levels for Radiochemical Assay

from the same nuclei and/or subsystems were pooled from different serial coronal sections of individual brains as well as across subjects of the same treatment group. Only tissues that could be easily identified, dissected, and isolated as relatively pure representations were saved for the radiochemical assay.

Coronal section 1 (Figure 3B) represented the telencephalon brain region and yielded the following individual tissues and/or systems (Saline Control vs. Pentobarbital): (a) fornix-septum-hippocampus (Pooled Tissue 1), (b) neostriatum (Pooled Tissue 2), and (c) cerebral cortex (Pooled Tissue 3).

Coronal section 2 (Figure 3C) represented the diencephalon and yielded (Saline Control vs. Pentobarbital): (a) thalamus (Pooled Tissue 4) and (b) hypothalamus (Pooled Tissue 5).

Coronal sections 3 and 4 (Saline Control vs. Pentobarbital) yielded the brain stem reticular formation-raphé nucleus-locus ceruleus (Pooled Tissue 6).

Coronal section 5 (Saline Control vs. Pentobarbital) yielded the cerebellum (Pooled Tissue 7).

Radiochemical assay. Pooled tissues/CNS region/ treatment group were then weighed and made into 10% (W/V) homogenates in glass-distilled deionized water. The homogenates were transferred to appropriately labelled 15 ml glass tube test tubes (Corex), placed into a Sorvall Angle centrifuge, and spun at 3000 r.p.m. for 15 min. The

supernatants (S1) were then poured into labelled scintillation counting vials. The sedimented pellets (P1) were solubilized by adding 1 ml Triton X-100 to each tissue sample test tube. The suspension was then thoroughly mixed in a Vortex mixer until a soapy mixture was obtained. These particulate fractions were then transferred to appropriately labelled scintillation counting vials. 10 ml of Biofluor liquid scintillation cocktail was added to each sample vial, and the vials were gently shaken. Vials were placed into a Delta 300 liquid scintillation counter (Searle Analytic Inc.) and the radioactivity of tritiated tissue samples was then counted (counts per min. [c.p.m.]) for 10 min./sample at 98% efficiency. A 5 µl aliquot of 5 nM [^{3}H]-TRH (specific activity 115.0 Ci/mmol) was transferred to a counting vial as a standard and 10 ml of Biofluor was added. The count rate (c.p.m.) of the standard was obtained in the scintillation counter concomitant with sample counting. A "noise" background count rate was also obtained by adding 10 ml of Biofluor to an empty vial, which was then placed in the scintillation counter. The background count rate was subtracted from the standard and all sample count rates to obtain corrected count rate values. Corrected sample values were compared to the corrected standard value to calculate soluble vs. particulate regional brain tissue concentrations of [^{3}H]-TRH.

Since the original <u>in vivo</u> administered (i.vt.) [^{3}H]-TRH concentration was 25 fmol/5 µl (5 µl of 5 nM), this value

was divided by the total brain tissue weight to obtain an estimated average tissue concentration level. The actual obtained average tissue concentration level was then divided by this latter value, and then the ratio was multiplied by 100 to obtain a percent recovery value. The recovery value was calculated from the average tritiation concentration rather than the total concentration because not all tissue was utilized in the radiochemical assay, i.e., only purely isolated tissues were used.

RESULTS

Experiment 1

The histology results indicated that there was a 90% level of precision (n = 20 mice brains) in the i.vt. procedure. Two representative photomicrographs are presented (Figures 4A and 5A) to illustrate how the violet dye has spread through the lateral ventricles. Coronal diagrams (Figures 4B and 5B) have been included to show the relationship between sites of dye and the lateral ventricles and surrounding CNS areas. Figure 6 shows that the right lateral ventricle can be obtained with the following mean ($\pm S_{\bar{x}}$) coordinates: (a) scalp--3.5 mm posterior to anguli oculi lateralis (at the anterior bregma level of the underlying skull), 1.0 mm right lateral to the midline, and 4.5 mm ventral to the scalp surface; (b) skull--0.5 mm ($\pm$0.1) anterior to posterior bregma, 1.0 mm ($\pm$0.1) right lateral to the midsagittal suture, and 4.5 mm ventral (which includes the 0.5 mm bevel edge of the 30 gauge LNR Yale needle).

Figure 4A is a photomicrograph of an i.vt. dye injection made at the exact coordinates listed above. As can be seen, the dye is clearly localized within the right lateral ventricle. Figure 5A is a photomicrograph of an i.vt. dye injection made 0.1 mm anterior to that of Figure 4A. A comparison of the histology slides (viewed under a light microscope) with corresponding photomicrographs confirmed that the site of the dye injection was localized within the right lateral ventricle. This is indicated in the photomicrograph

Figure 4A. Photomicrograph (10x) showing site of intraventricular (i.vt.) dye (Harris' hematoxylin) injection. Note prominence of violet dye within the choroid plexus of the right lateral ventricle of the CF-1_S mouse brain at the skull coordinates +0.5mmA, +1.0 mmL_r, and 4.5 mmV relative to posterior bregma.

Figure 4B. Diagram of a coronal section indicating the relationship between the site of the violet dye in the photomicrograph with the location of the lateral ventricles and surrounding brain regions: (1) right lateral ventricle (site of violet dye i.vt injection), (2) neocortex, (3) caudate nucleus/putamen, (4) corpus callosum, (5) medial septal nucleus, (6) nucleus accumbens septi, and (7) preoptic suprachiasmatic nucleus.

Figure 4A.

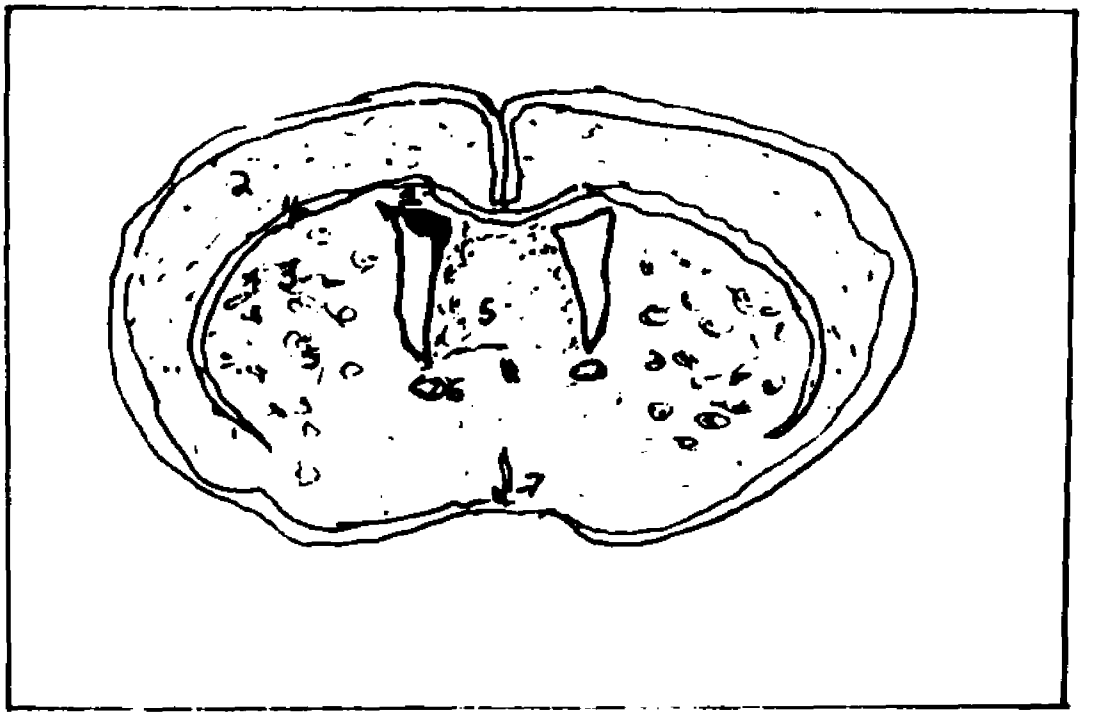


Figure 4B.

Figure 5A. A photomicrograph of a coronal section from the CF-1_S mouse brain (10x) illustrating that the violet Harris' hematoxylin dye has diffused away from its original right lateral ventricle site towards the left lateral ventricle. The coordinates of the i.vt. injection were the same as Figure 4A, except 0.1 mm anterior. The dye spread to 1.0 mm anterior and -3.0 mm posterior relative to the original injection site.

Figure 5B. Diagram of a coronal section indicating the relationship between the site of the violet dye in the photomicrograph with the location of the lateral ventricles and surrounding brain regions: (1a) right lateral ventricle (site of violet dye i.vt. injection), (1b) left lateral ventricle (site of diffused violet dye in photomicrograph), (2) neocortex, (3) caudate nucleus/ putamen, (4) corpus callosum, (5) medial septal nucleus, (6) nucleus accumbens septi, and (7) preoptic supra-chiasmatic nucleus.

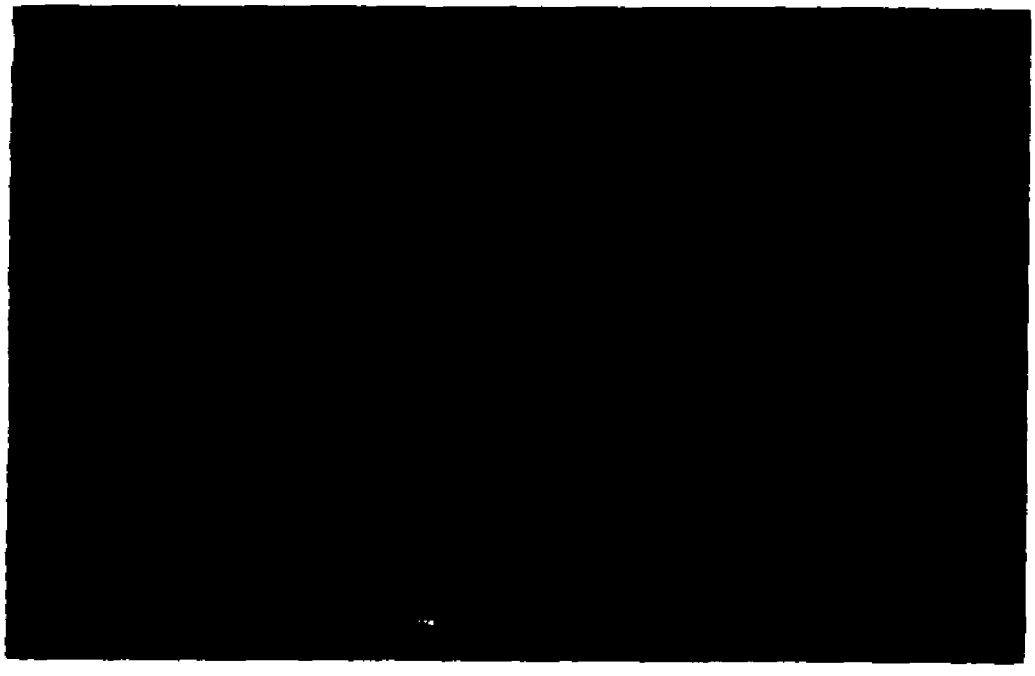


Figure 5A.

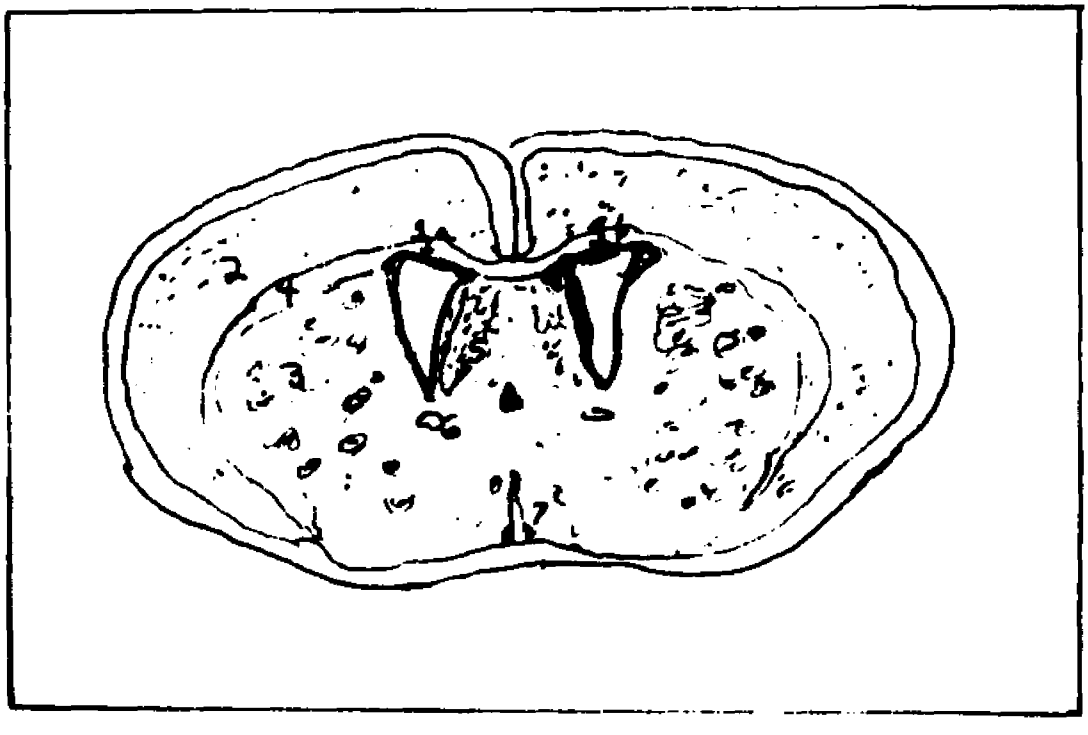


Figure 5B.

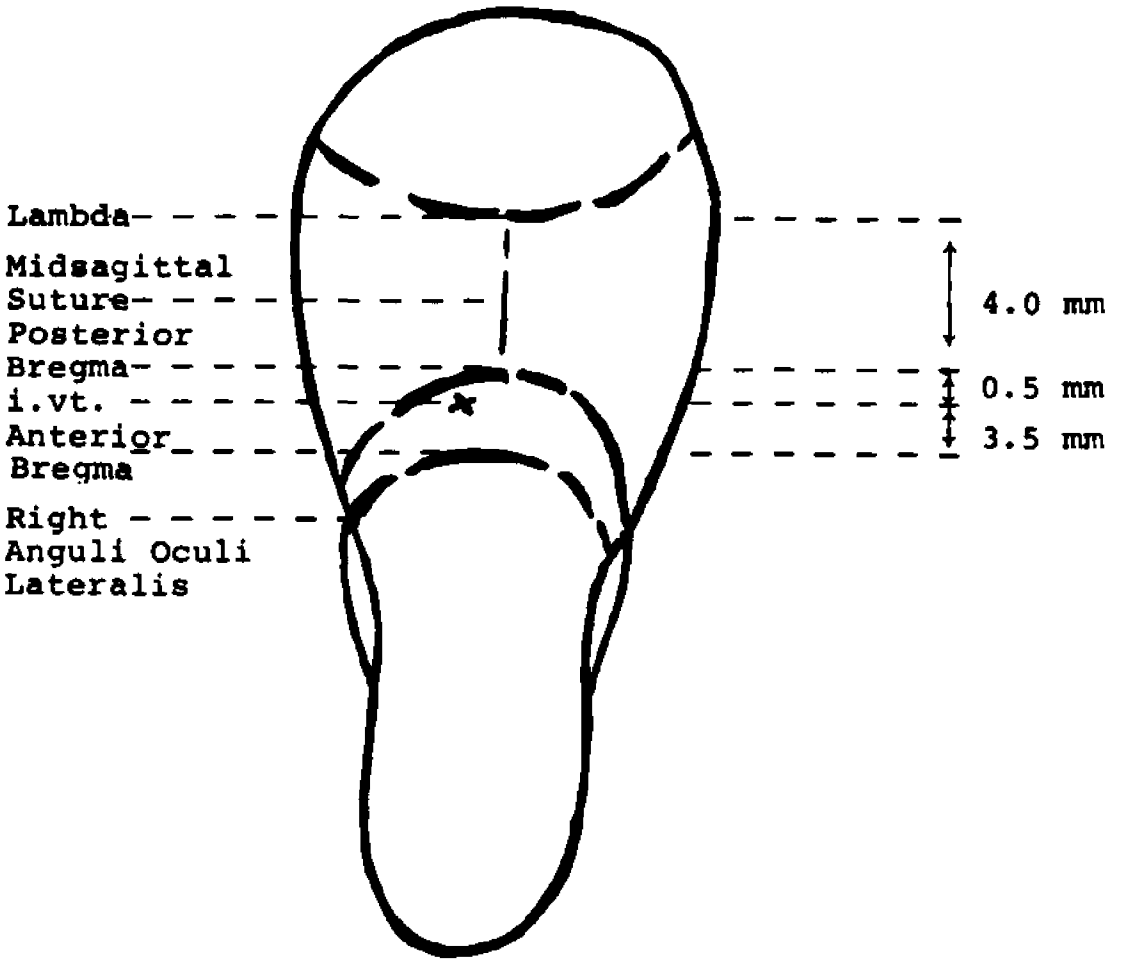


Figure 6. Diagram showing coordinates used for microsyringe intraventricular (i.vt.) injection into the CF-1$_S$ mouse right lateral ventricle (Experiment 1). Mean scalp coordinates were 3.5 mm posterior to anguli oculi lateralis (at anterior bregma level of the underlying skull), 1.0 mm lateral to midline, and 4.5 mm ventral. The mean skull coordinates were 0.5 mm ($S_{\bar{x}}$ = 0.1) anterior to posterior bregma, 1.0 mm ($S_{\bar{x}}$ = 0.1) right lateral to the midsagittal suture, and 4.5 mm ventral. The precision of the i.vt. procedure utilizing these coordinates was 90% (n = 20).

(Figure 5B) by the prominence of dye within this brain region. This photomicrograph is being presented to demonstrate that the dye can diffuse throughout the ventricular system. The two lateral ventricles communicate with one another and the III ventricle through the interventricular foramen (Williams & Warwick, 1975). The photomicrograph displays the abundance of dye diffusion to the left lateral ventricle. The dye also diffused as anterior (A) as $\pm$1.0A and as posterior (P) as -3.0P (relative to posterior bregma) with dye located in the III ventricles and the hippocampus as well (not shown).

Experiment 2

The results of this factorial experiment indicated that both TRH and pentobarbital have profound effects with regard to narcosis in three month old male CF-1_S mice: As is apparent in Table 7, the *F* values for both drug dose effects were significant at the $p < .01$ significance level. While this was the criterion level chosen, a second analysis of variance (via BMDP2V computer program, U.C.L.A., 1979) of this 3 X 3 factorial experiment as a 2 X 3 factorial experiment (since Barbiturate Level I has no variance) indicates that both *F* values were actually significant at the $p < .001$ level. The low *F* value (1.22 [2, 54 df]) for the TRH-barbiturate interaction factor was not significant (not shown). A priori orthogonal *t*-tests (Table 7) indicate that pentobarbital has marked dose-response actions in the induction of narcosis (duration of effect). Further, the *t*-tests

Table 7

Mean Duration of Barbiturate Narcosis (min.)

Factorial Design: Experiment 2

			TRH[a] Dose (i.vt.)		
			0 µg	10 µg	20 µg
Pentobarbital[b] Dose (i.p.)	(I)	0 mg/Kg	0	0	0
	(II)[c]	50 mg/Kg	120.5 (8.3)	58.7 (6.9)	57.0 (5.2)
	(III)[d,e]	70 mg/Kg	239.4 (14.5)	157.6 (14.9)	141.5 (14.2)

Note. n = 10 per cell; values in parentheses are Standard Errors of the Mean.

[a] $F_{TRH\ Dose} = 30.19$, $p < .01$ (2,54 df)

[b] $F_{Pentobarbital\ Dose} = 117.52$, $p < .01$ (1,54 df)

[c] $t_1 = 4.47$, $p < .01$ (54 df), Mean TRH_{II} vs. Saline $Control_{II}$

[d] $t_2 = 6.46$, $p < .01$ (54 df), Mean TRH_{III} vs. Saline $Control_{III}$

[e] $t_3 = 10.84$, $p < .01$ (54 df), Mean $Pentobarbital_{II}$ vs. Mean $Pentobarbital_{III}$

indicate that TRH significantly reduces the duration of the pentobarbital-induced narcosis at both high (70 mg/Kg) and low (50 mg/Kg) barbiturate levels (Table 7 and Figure 7). At the low barbiturate levels, 10 μg and 20 μg TRH significantly reduced narcosis duration by 52% compared to the saline (0 μg) control ([saline narcosis - TRH narcosis/saline narcosis] x 100); at the high barbiturate level, 10 μg and 20 μg TRH reduced narcosis to 41% of the control baseline level.

On the other hand, Figure 7 indicates that the central TRH analeptic effect is non-dose related. A priori t-tests (not shown) indicated that at both high (Level III) and low (Level II) barbiturate levels, effects for 20 μg TRH did not significantly differ from 10 μg. This is shown by the two "breaks" in the TRH curve (Figure 7). This phenomenon suggests possible toxic or spare receptor effects. This viewpoint will be clarified later (see Discussion). TRH administrations alone to non-anesthetized mice did not display inductions of narcosis or any other observable behavioral effects.

Experiment 3

A BMDP2V computer program analysis of variance of the split-plot factorial data indicated that there were marked significant main and interaction effects between central (i.vt.) TRH dose and the interinjection duration (IID) at the $p < .01$ significance level (Table 8 and Figure 8). The computer program indicated that all three F-test comparisons

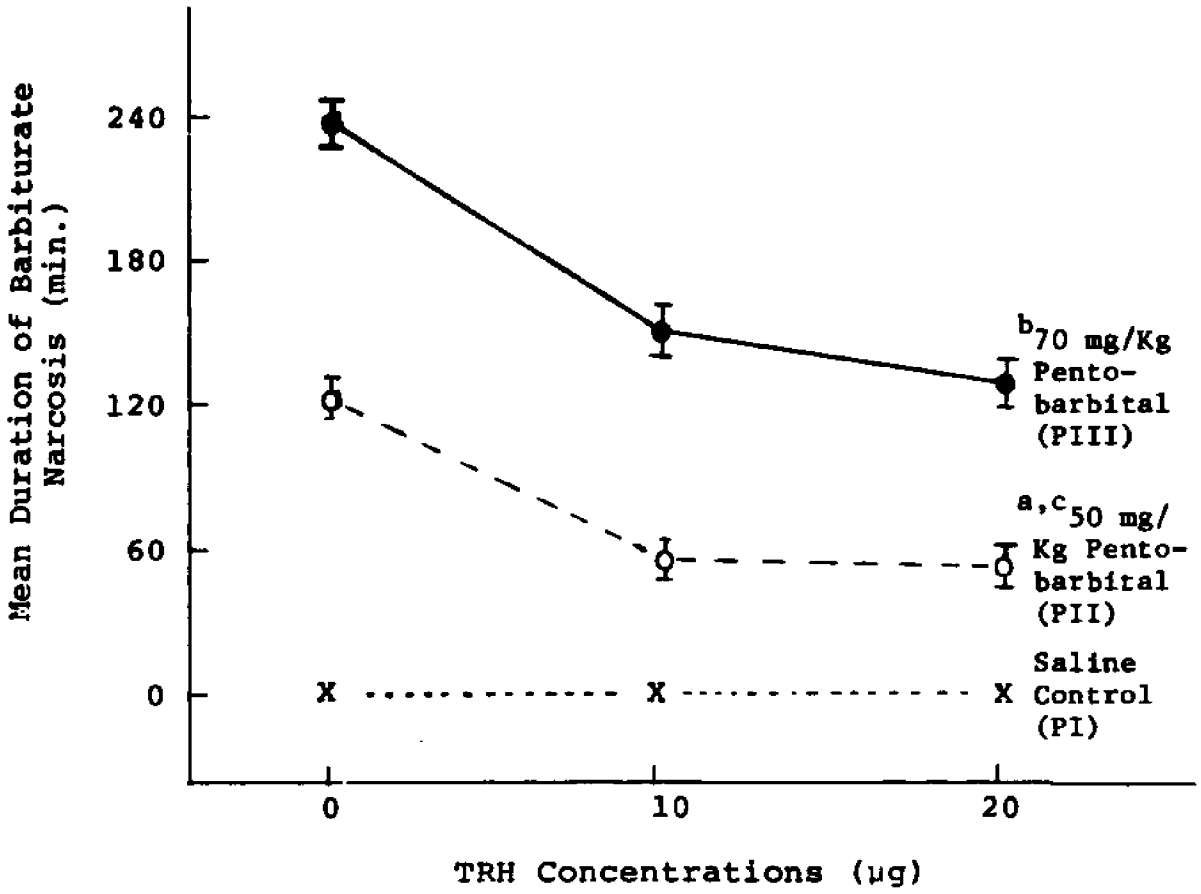


Figure 7. Dose-response curve of TRH antagonism of pentobarbital-induced narcosis in three month old male CF-1_S mice (Experiment 2). TRH administered (i.vt.) 1 min. before pentobarbital (i.p.) significantly reduced barbiturate narcosis in a non-dose related manner, indicating possible toxic or spare receptor effects.

[a] $p < .01$ $\overline{TRH}$ vs. Saline Control (PII), ($\pm_{.01/2,\ 54\ df} = 4.47$).

[b] $p < .01$ $\overline{TRH}$ vs. Saline Control (PIII), ($\pm_{.01/2,\ 54\ df} = 6.46$).

[c] $p < .01$ $\bar{P}II$ vs. $\bar{P}III$, ($\pm_{.01/2,\ 54\ df} = 10.84$).

I = Standard Error of the Mean.

Table 8

Mean Duration of Barbiturate Narcosis (min.)

Split Plot Factorial: Experiment 3

		Interinjection Duration (IID)[b]		Sum Score[c]	Difference Score[d]
		1 min.	45 min.	(Σ)	(Δ)
TRH Dose[a] in µg	0	85.0 (5.3)	81.9 (5.5)	166.9	3.1
	10	50.5 (2.9)	68.9 (2.1)	119.4	18.4
	20	34.3 (1.5)	66.6 (4.6)	100.9	32.3

Note. 50 mg/Kg pentobarbital was a parameter; n = 20 per cell; values in parentheses are Standard Error of the Mean.

[a] F_{Dose} = 28.01, $p < .01$ (2,48 df).

[b] F_{IID} = 36.29, $p < .01$ (1,48 df).

[c] $t_{Dose(\Sigma)}$ comparisons: t_{12} = 7.38, $p < .01$ (48 df); t_{13} = 10.25, $p < .01$ (48 df); t_{23} = 2.87, $p < .01$ (48 df); [1 = 0µg, 2 = 10µg, 3 = 20µg TRH].

[d] $F_{Dose \cdot IID}$ = 15.32, $p < .01$ (2,48 df); Scheffé $t_{Dose \cdot IID(\Delta)}$: t_{12} = 5.62, not sig. (48 df); t_{13} = 20.50 $p < .01$ (48 df); t_{23} = 4.64, not sig. (48 df); [1 = 0µg, 2 = 10µg, 3 = 20µg TRH].

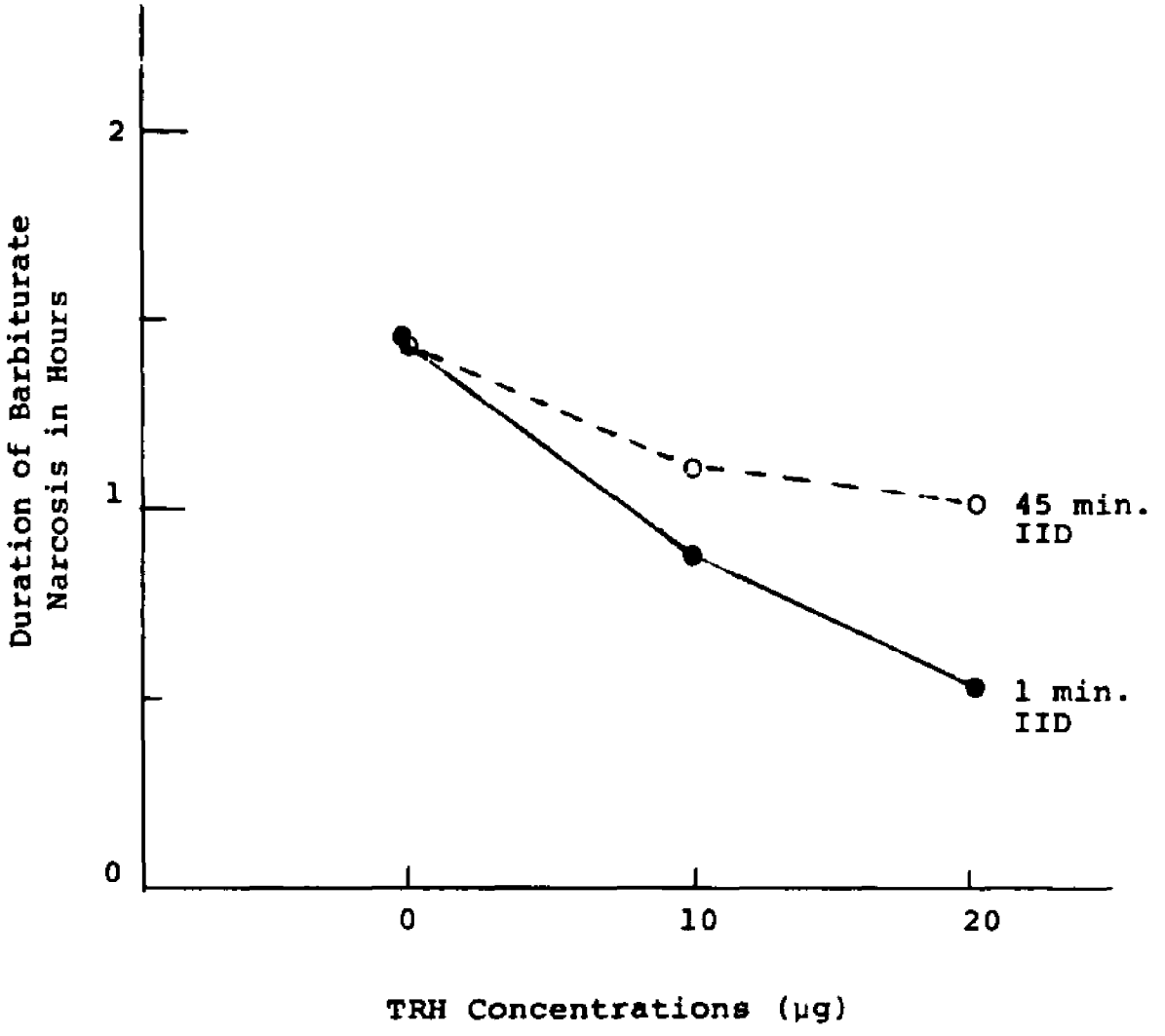


Figure 8. Graph showing the effects of TRH-Interinjection Duration (IID) interactions upon the TRH analeptic mechanism (Experiment 3). At a 1 min. IID, TRH displays a dose-response relationship. However, at a 45 min. IID, the analeptic effect is attenuated.

were actually significant at the $p < .0001$ level. A posteriori t-tests (using $MS_{\text{subjects within groups}}$ as the error term) for the main TRH effect (Sum Scores, Table 8) showed that the analeptic effect was dose-related from 0-20 μg. Scheffé t-tests of the TRH-IID interactions (Difference Scores, Table 8) indicated that the only significant interaction effects ($t_{13} = 20.50$, $p < .01$ [2, 48 df]) were between the 20 μg TRH and 0 μg TRH (saline) dose levels: The difference score between narcosis durations at 1 min. and 45 min. IIDs for 20 μg TRH was large, while the difference score for 0 μg was small. Thus, the higher TRH dose was the most sensitive to IID interactions. As illustrated in Figure 8, TRH displays a dose-response relationship when TRH is administered immediately before pentobarbital (1 min. IID). However, when the peptide is centrally administered 45 min. before the barbiturate, there is no dose-response relationship, i.e., 20 μg TRH actions are not significantly different from 10 μg TRH actions which are in turn greatly attenuated towards the saline control baseline level.

In addition, the analysis of variance results indicated that there were no diurnal variations in the central TRH effect. The F-test value for this variable was not signicant, nor were there any other significant factor interactions with this variable. Thus, the peptide appears to be equally effective as an analeptic in both the A.M. and P.M. phases of the day. Also, the analysis of variance results indicated no significant order effects ($p < .01$) for repeated

measures of treatments (F = 5.19 [1,48 df]). Additionally, there were no significant interactions between the order factor and any other factors including TRH dose levels.

Experiment 4

The biochemical assay results from Experiment 4 showed that TRH does not alter mouse brain levels of pentobarbital (Table 9). The mean level of both whole brain as well as regional concentrations 1 hour after barbiturate (i.p.) injections were within the close range of 18.0-20.2 μg/g tissue for all treatment conditions. An analysis of variance of whole mouse brain barbiturate levels indicated no significant differences between central (i.vt.) 20 μg TRH vs. saline control pretreatments, nor between IID effects. Further, correlated t-tests within treatments indicated no significant differences (at $p < .01$) between regional barbiturate levels. Thus, it would appear the TRH's analeptic actions are not related to peptide-induced alterations of brain concentration of pentobarbital. The results of the pentobarbital assay can be considered as precise, since it was found to yield a 90% recovery of an exogenous pentobarbital standard run through the procedure.

Experiment 5

Despite the relative closeness in body weight and age between two month old vs. three month old male CF-1_S mice, there appeared to be distinct differences in drug sensitivities between the two groups (Table 10): A 2^2 analysis of variance (via a BMDP2V program) of data regrouped from

Table 9

Effects of Central (i.vt.) TRH Administrations Upon Pentobarbital Disposition in the CF-1_S Mouse Brain

	Mean Brain Concentrations of Pentobarbital (µg/g Tissue)	
Whole Brain[a]	Saline	20 µg TRH
Interinjection Durations (IID)		
1 min.	18.9 (0.7)	19.5 (0.7)
45 min.	19.9 (1.0)	19.3 (1.1)
Regional (1 min. IID)[b]		
1. Telencephalon	18.9 (0.3)	19.5 (0.6)
2. Diencephalon	19.6 (0.6)	20.2 (0.5)
3. Brain Stem	18.0 (0.6)	18.9 (0.6)

Note. n = 5 (Σ3 pooled)/cell; ( ) = $S_{\bar{x}}$; 50 mg./Kg pentobarbital i.p. = parameter; 90% recovery/assay.

[a] 2 x 2 ANOVAR: No signif. $p < .01$ in [pento.] bet. (a) treatments or (b) IIDs (F = 0.27; 3,16 df); 1 min. IID = $\bar{X}$ 3 regions/sample x 5.

[b] t-tests correl./treatment$_i$: No signif. ($p < .01$, 4 df) bet. regional [pento.]: t_{12S} = 1.21, t_{13S} = 2.82, t_{23S} = 3.84; t_{12T} = 1.71, t_{13T} = 1.97, t_{23T} = 1.97; S = Saline, T = TRH.

Table 10

The Effects of Age on the TRH Analeptic Mechanism.

Mean Duration of Barbiturate Narcosis (min.)

Age	Drug Treatment Order			
	Pre[a] TRH Dose		Post[b] TRH Dose	
	0 µg	20 µg	0 µg	20 µg
2 Months	92.7 (7.3)	30.9 (1.6)	77.3 (4.5)	56.6 (5.1)
3 Months	120.5 (8.3)	57.9 (5.2)	127.8 (9.1)	99.0 (8.8)

Note. n = 10 per cell; values in parentheses are Standard Errors of the Mean.

[a]Pre = TRH or saline (i.vt.) at 1 min. before 50 mg/Kg pentobarbital (i.p.) (Regrouped Data from Experiments 2 and 3). 2^2 Analysis of Variance: F_{age} = 19.85, significant $p < .01$ (1,36 df); F_{TRH} = 102.27, significant $p < .01$ (1,36 df); $F_{TRH \cdot age}$ = 0, not significant (1,36 df).

[b]Post = TRH or saline (i.vt.): 1 min. after 50 mg/Kg pentobarbital (i.p.) plus at 5 min. after loss of the righting reflex. 2^3 Analysis of Variance (Experiment 5): F_{age} = 38.11, significant $p < .005$ (1,32 df); F_{TRH} = 10.84, significant, $p < .005$ (1,32 df); $F_{post\ IID}$ = 0.06, not significant (1,32 df).

Experiments 2 and 3 revealed that both TRH dose and age have a significant influence upon barbiturate narcosis. Central (i.vt.) administrations of TRH at 1 min. before 50 mg/Kg of pentobarbital (i.p.) significantly reduced barbiturate narcosis by 66.7% in two month old mice, and by 52% in three month olds. While a $p < .01$ significance level was utilized for statistical testing, the BMD program indicated that the F-values for these two pharmacological agents were outside the $p < .0001$ significance level. The results showed that the age factor does not interact with TRH dose.

In Experiment 5, 20 μg central TRH administrations following barbiturate injections were again effective in antagonizing the drug-induced narcosis. Both F_{TRH} dose and F_{age} were significant at $p < .005$ levels. However, the "Post" TRH analeptic actions reduced the mean barbiturate narcosis by only 22.5-26.8% from saline control levels. This can be contrasted with the 52-67% analeptic actions for "Pre" TRH administrations. A comparison of the two "Post" IID's showed no significant differences ($F = 0.06$, [1,36 df]) between TRH infusions at 1 min. after barbiturate injections vs. infusions at 5 min. after initial narcosis induction (time at which righting reflex is first lost). Also, the IID factor did not significantly interact with any other variables. The age factor for "Post" conditions did not significantly interact with any other variables, including TRH dose.

Experiment 6

The results of the biochemical assay are summarized in

Table 11. The data indicate that there are significant differences in regional brain barbiturate levels between CF-1_S mice of two and three months of age at 1 hr. following barbiturate administrations in combination with either TRH or physiological saline pretreatments. TRH did not alter regional brain barbiturate concentrations in comparison to saline baseline levels. Within age groups, the regional CNS pentobarbital concentrations were fairly uniform.

Experiment 7

Table 12 is a summary listing of the *in vitro* [^{3}H]-TRH radioreceptor results: The highest concentrations of TRH binding sites in the CF-1_S mouse brain were in the limbic forebrain area. This is a rather widespread region and includes the hippocampus, septum, fornix, neostriatum, hypothalamus, and the thalamus. The K_D for postsynaptic membrane receptor sites of this CNS area was 34.1 nM, and thus displayed a slightly greater affinity for TRH than the presynaptic membrane receptors with a K_D of 45.4 nM. The high concentration of specific TRH binding sites were found to be 185.8 fmol/mg protein for the former sites and 195.1 fmol/mg protein for the latter, which were 75% and 69%, respectively, of the total binding.

The brain stem region--which included the mes-, met-, and myelencephalon--was also found to have an extremely high density of specific TRH binding sites. While there were no detectable postsynaptic receptors in this CNS area, there was an abundance of presynaptic receptors: The 166.6

Table 11

Altered Brain Barbiturate Levels

as a Function of Age

Age Group	Pretreatment	Mean Regional CNS Concentrations of Pentobarbital (μg/g)[a,b]		
		Telencephalon	Diencephalon	Brain Stem
2 months	TRH	9.6 (0.2)	10.0 (0.2)	9.4 (0.5)
	Saline	9.8 (0.2)	9.9 (0.1)	9.7 (0.1)
3 months	TRH	16.4 (0.3)	16.1 (0.2)	15.9 (0.3)
	Saline	15.9 (0.3)	15.6 (0.5)	15.8 (0.4)

Note. n = 3/cell; 50 mg Na pentobarbital/Kg (i.p.) was a parameter. 20 μg TRH or physiological saline was administered (i.vt.) at 1 min. before barbiturate injections. $S_{\bar{x}}$ in parentheses.

[a]Analysis of Variance: $F_{treatments}$ = .18, not sig. (1, 24 df); $F_{regions}$ = .34, not sig. (2, 24 df); F_{age} = 761.79, $p < .001$ (1, 24 df).

[b]Scheffé t-tests indicate significant age differences ($p < .01$, 5 df); no significant TRH effects ($p < .01$, 5 df); and no signficant regional differences within age groups ($p < .01$, 2 df).

Table 12

Regional Distribution of TRH Receptor Binding and Barbiturate Competitive Inhibition in the CF-1_S Mouse Brain: *In vitro* [^{3}H]-TRH Radioreceptor

Brain Region	Synaptic Receptor Membrane Preparation	High Affinity Binding K_D (nM)	High Affinity Binding # Specific TRH Binding Sites (fmol/mg Protein)	High Affinity Binding % of Total Binding	Kinetics % Dissoc. (45 min.)	Competitor	% TRH Inhibition
I. Cerebral Cortex	(1) Pre-	-	0	0	-	-	-
	(2) Post-	126.3	145.1	44	-24	Pheno	-242
						Pento	-225
						Thio	-243
II. Limbic Forebrain	(1) Pre-	45.4	195.1	69	+12	Pheno	- 31
						Pento	+ 57
						Thio	+ 45
	(2) Post-	34.1	185.8	75	+33	Pheno	+ 96
						Pento	+ 63
						Thio	+ 61
III. Brain Stem	(1) Pre-	9.4	166.6	91	-14	Pheno	+100
						Pento	+100
						Thio	+100
	(2) Post-	-	0	0	-	-	-

fmol/mg protein binding site density for this area--which was 91% of the total binding--was relatively close to the high receptor densities of the limbic forebrain region. Also, these brain stem receptors displayed the highest affinity for TRH--with a K_D of 9.4 nM--a value which was 4-5 orders of magnitude lower than the limbic forebrain K_D.

The radioreceptor assay did not detect any discernible specific TRH binding at presynaptic membranes of cerebral cortex tissue origin. There was, however, a fairly high density of postsynaptic TRH membrane receptors in this area. Nonetheless, the 44% specific binding density was lower than for any other CNS region; these receptors displayed a K_D of 126.3 nM, a value 3-4 times lower in affinity, i.e., higher in dissociation, than that displayed by limbic receptors, and 13 times lower in affinity than that displayed by brain stem presynaptic receptors.

The TRH kinetic assay data indicated that only the limbic forebrain receptors displayed true [^{3}H]-TRH dissociations (+% values) within the 45 min. incubations. However, even these receptors displayed only 12-33% dissociations, suggesting possible positive cooperativity effects. Both the postsynaptic cortical receptors and the presynaptic brain stem receptors did indeed display these cooperative effects since there were 24% and 14% increases (negative dissociation values), respectively, in specific TRH binding over the kinetic assay period.

The competition of barbiturate analogues for TRH

receptors closely matched the degree of specific TRH binding to these same receptors: The brain stem presynaptic sites, which displayed the highest affinity for TRH, also displayed the highest barbiturate competitive inhibition of TRH. All three analogues--phenobarbital, pentobarbital, and thiopental--displayed 100% TRH inhibition when competed with the peptide at these specific high-affinity receptors. The barbiturate analogues were also found to be effective competitive inhibitors at both presynaptic and postsynaptic limbic forebrain receptors, showing 45-96% inhibitions at these sites. The postsynaptic receptors of this region appeared to have a somewhat higher affinity than presynaptic receptors for the barbiturates. The cerebral cortex--which had the lowest density of specific TRH receptors, the lowest regional affinity for the peptide, and highest display of positive cooperativity effects (in the TRH dissociation kinetics assay)--also displayed extremely marked positive cooperative effects in the specific peptide binding assay following incubations of these postsynaptic receptors with any of the barbiturate analogues. All three analogues displayed -225% to -243% competitive inhibitions of TRH, thus indicating that these drugs actually augment TRH binding at their specific cortical binding sites.

The [^{3}H]-TRH radioreceptor data were statistically analyzed for regional brain differences in barbiturate competitive inhibition. A 2 x 3 Median Test comparing CNS area with type competition, i.e., "true (+) competition" vs.

"positive cooperativity" and/or no competition, indicated that there was a highly significant association between region and differences in barbiturate competition (χ^2 [2 df] = 8.7, $p < .02$).

Experiment 8

The *in vivo* [^{3}H]-TRH radiochemical assay results (Table 13 and Figure 9) are in close agreement with the *in vitro* radioreceptor results of Experiment 7. Centrally (i.vt.) infused [^{3}H]-TRH redistributed within 1 hour from the right lateral ventricles of physiological saline-treated (i.p.) mice to limbic forebrain structures: 38% of the total centrally diffused tritiated peptide was localized within the septal-fornix-hippocampus complex ("hippocampal system"), and 21% was localized within the hypothalamus. The highest cellular particulate-bound tritiated TRH was localized within the hypothalamus which contained 31% of the total brain peptide distribution in saline treated mice. The hippocampal system also contained 23% of the total particulate-bound peptide. The thalamus contained 5% of total tritiation (soluble plus particulate), and only 17% of total particulate-bound tritiation. The cerebral cortex contained the least amount of total tritiation--3%--and did not contain any particulate-bound tritiation. The entire brain stem region contained about 5% of the total tritiation, and about 12.5% of particulate-bound tritiation (although the cerebellum contained less than one-half this amount).

The effects of parenteral (i.p.) pentobarbital

Table 13

Regional Mouse Brain Distribution of [^{3}H]-TRH After Central Administrations: Competition With Barbiturates for Active Sites

CNS Region	Fraction*	[^{3}H]-TRH Concentrations (amoles/mg original tissue) Saline Control	Pentobarbital
1. Septal-Hippocampus	S	84.9	28.7
	P	8.7	1.6
	Σ	93.6	30.3
2. Basal Ganglia	S	54.0	40.4
	P	3.6	3.2
	Σ	57.6	43.6
3. Cerebral Cortex	S	7.6	28.4
	P	0	2.9
	Σ	7.6	31.3
4. Thalamus	S	5.4	7.2
	P	6.4	6.4
	Σ	11.8	13.6
5. Hypothalamus	S	41.3	11.1
	P	11.8	3.5
	Σ	53.1	14.6
6. Brain Stem	S	7.9	12.6
	P	4.7	5.0
	Σ	12.6	17.6
7. Cerebellum	S	9.3	10.1
	P	2.3	4.7
	Σ	11.6	14.8

Note. 5 µL of 5 nM [^{3}H]-TRH (i.vt.) at 1 min. pre-saline vs. 50 mg/Kg pentobarbital. χ^2 tests: Regions x Treatments = Signif. $p < .01$ (6 df); Fractions x Treatments = Signif. $p > .95$ (1 df).

*S = Soluble; P = Particulate

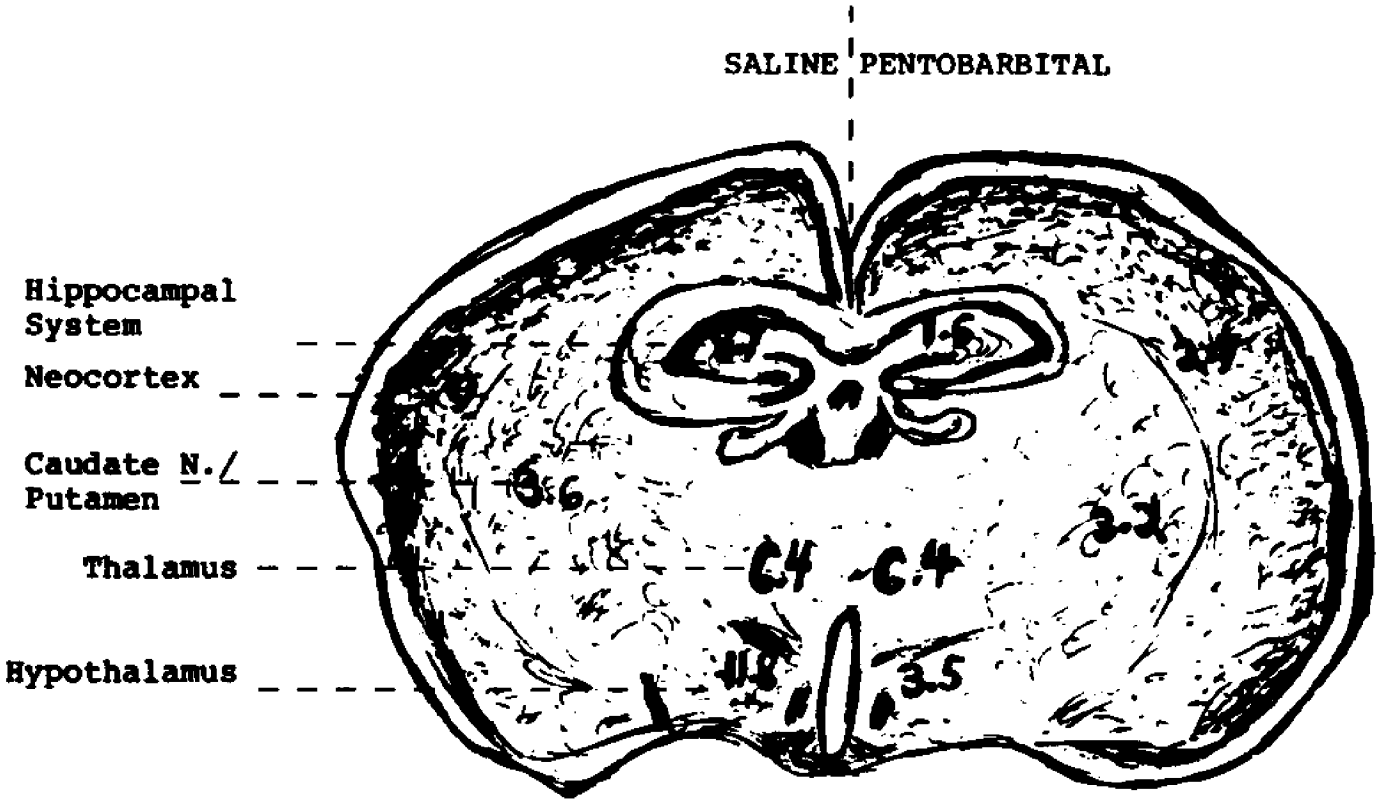


Figure 9. Coronal Section Illustrating In Vivo Regional Distribution of [^{3}H]-TRH in CF-1$_S$ Mouse Brain After i.vt. Administrations. Values on Left (amol/mg Original Tissue) are for Saline Challenge; Values on Right are for Pentobarbital Challenge.

inductions upon the mouse brain distribution of [^{3}H]-TRH were quite profound: The total tritiation of the hippocampal system was reduced to < 50% of its saline baseline level following barbiturate treatments (from 38% to 18%, respectively), and its particulate-binding was reduced by approximately 75% below saline baseline levels (from 23% to 6%, respectively). The hypothalamus also displayed profound changes under these conditions: Total tritiation was reduced to < 43% of its saline baseline levels at 1 hour following barbiturate inductions (from 21% to 9%, respectively), and particulate-binding was also reduced to < 43% of baseline following this drug administration (from 31% to 13%, respectively). The basal ganglia, which had a relatively high initial total and particulate-bound [^{3}H]-TRH concentration after parenteral saline injections (23% of total tritiation and 10% of particulate), maintained these levels after barbiturate treatments. Thus, this CNS region would not appear to be involved in TRH-barbiturate interactions. The thalamus showed a similar pattern, as did the brain stem.

It should be mentioned that total tritiation did increase in this latter area following barbiturate treatments, although particulate-bound levels were relatively invariant. It is possible that the positive cooperative receptor effects within this region are responsible for the 160% increase in saline baseline soluble [^{3}H]-TRH concentrations following barbiturate treatments. The cerebral cortex also displayed this same interesting phenomenon. 20% of total brain

tritiation was distributed in this area following central [^{3}H]-TRH administrations to saline-treated mice, but 0% of particulate-bound tritiation was found in this area. On the other hand, although total tritiation levels were unchanged in this CNS region following barbiturate treatments, the particulate-binding suddenly increased from the 0% level to almost 11% after administration of the anesthetic. Again, this phenomenon might be related to the extremely high positive cooperativity receptor effects found in post-synaptic receptors of this area.

The *in vivo* radiochemical data were analyzed by two chi-square (χ^2) tests to further test the hypotheses that: (a) TRH has a unique non-uniform central distribution, and (b) that barbiturate displaces tritiated TRH from these unique particulate-bound sites in a mode similar to its [^{3}H]-TRH radioreceptor competitive inhibition mechanism. A 7 x 2 χ^2 test of CNS regions x drug treatments revealed that the two factors were strongly associated with one another since the null hypothesis of no relationship was rejected at $p < .01$ (6 df). The second hypothesis was also supported since a 2 x 2 χ^2 test of cellular fraction (particulate vs. soluble) x drug treatments rejected the null hypothesis of no association for these two factors at $p > .95$ (1 df). Thus, it can be concluded: (a) that *in vivo* TRH redistributes following its central (i.vt.) administration, and then becomes localized within specific synaptosomal particulate binding sites of the limbic forebrain, and (b) that

barbiturates interact at these same sites to displace the peptide from its unique and specific central binding sites. The *in vivo* [^{3}H]-TRH radiochemical data thus tend to validate the *in vitro* [^{3}H]-TRH radioreceptor findings.

DISCUSSION

This project has been concerned with a further clarification of the central processes regulating TRH antagonism of barbiturate narcosis. It is proposed that the analeptic mechanism of TRH is mediated at discrete TRH binding sites. A series of experiments have been performed to test several proffered hypotheses possibly related to different parallel levels of this phenomenon. In the following discussion, the present findings will be considered under the specific topics of behavioral pharmacology, biochemistry, and neurochemistry-neurophysiology to facilitate integration of the accrued information into the framework of knowledge developed in this area.

Behavioral Pharmacology

All of the pharmacological studies utilized the free-hand i.vt. procedure for centrally administering TRH into the mouse brain. The results of Experiment 1 support the reliability of this procedure. The same scalp and skull coordinates were utilized throughout this project. The first hypothesis proposed that TRH would display dose-related functions in the analeptic effect by this route of administration due to an avoidance of problems inherent in peripheral routes of administration, i.e., plasma and gastrointestinal enzyme degradation of peptide as well as difficult transport across the blood-brain barrier (Bissette et al., 1976; Eskay et al., 1976; Prange et al., 1974, 1975; Stumpf & Sar, 1973). The analysis of variance results of

Experiment 2 indicate that there were significant TRH dose effects when administered one minute before pentobarbital (i.p.) (Table 7). A priori orthogonal t-tests further indicated that mean TRH effects per pentobarbital dose level (50 mg/Kg or 70 mg/Kg) significantly reduced pentobarbital-induced narcosis below saline baseline levels. F-test and a posteriori t-test results of Experiment 3 (Table 8) further support Hypothesis 1: All three TRH dose-response comparisons--0 µg, 10 µg, and 20 µg--were significantly different from one another in a monotonically increasing order. This dose-response relationship is displayed in Figure 8.

On the other hand, the a priori t-tests of Experiment 2 also indicated that 20 µg TRH effects were not significantly different from 10 µg TRH effects in this paradigm (Table 7). Also, the F-test analysis for the TRH dose-barbiturate dose interaction effect was not significant. These phenomena are displayed in Figure 7. This graph illustrates that while both 10 µg and 20 µg TRH significantly reduce the durations of narcosis at both high vs. low barbiturate levels, the dose-response relationship "breaks down" beyond the 10 µg TRH limit. Why are there dose-response TRH analeptic effects in Experiment 3, but not in Experiment 2?

Age factors. The first point to be noted is that in Experiment 2 the mice were three months of age, while in Experiment 3, they were two months of age. A comparison of results from both experiments indicates that 20 µg TRH at

one minute before 50 mg/Kg pentobarbital reduced the duration of narcosis to a greater degree in two month old mice than in three month old mice (Table 10). The data from these two experiments were reanalyzed in Experiment 5 (using only the first session of one minute IID data from Experiment 3 for the two month old mice): The F-test value for the age effect was significant at the $p < .01$ significance level. The age factor again proved to be significant in the same direction when analyzed for effects of TRH post-treatment administrations (Experiment 5). These latter effects were attenuated (22.5-26.8% reduction of baseline narcosis durations) compared to the TRH pretreatment conditions. Thus, although not anticipated, it appears that age was an important relevant variable in the TRH analeptic effect. On the other hand, the analysis of variance results for age-TRH dose interactions in the combined Experiments 2 and 3 data (Table 10) reveal a lack of significance for this effect.

A further comparison of the barbiturate baseline data (50 mg/Kg pentobarbital plus 0 μg TRH) between these two age groups (Table 10) perhaps sheds some light on the discrepancy between the findings of significant age factors vs. a lack of significant age-TRH dose interactions: In Experiment 2, the three month age group of mice displayed a mean ($\pm S_{\bar{x}}$) baseline narcosis duration of 120.5 ($\pm$8.3) min., while the two month olds of Experiment 3 displayed a mean ($\pm S_{\bar{x}}$) baseline duration of 92.7 ($\pm$7.3) min. Thus, three month old mice were more sensitive to pentobarbital actions. The TRH

post-treatment data of Experiment 5 also match this pre-treatment data. Three month olds displayed a mean ($\pm S_{\bar{x}}$) baseline narcosis duration of 127.8 ($\pm$9.1) min., while two month olds displayed a mean ($\pm S_{\bar{x}}$) baseline duration of 77.3 ($\pm$4.5) min. Thus, the pretreatment data indicate that three month olds were anesthetized for 130% times the duration displayed by two month olds, and the post-treatment data indicate a 165% ratio in this same direction.

This age-related barbiturate sensitivity phenomenon can be contrasted with the age-related TRH phenomenon noted earlier: Two month old mice were more sensitive than three month olds to 20 µg TRH (i.vt.) actions whether administered before or after pentobarbital. Pre-treatments vs. post-treatments with TRH yielded 66.7% and 26.8% reductions, respectively, in baseline barbiturate narcosis durations for two month olds; these same conditions yielded 52.0% and 22.5% reductions, respectively for three month olds. Thus, three month olds are more sensitive to barbiturate-induced narcosis actions and the two month olds are more sensitive to TRH analeptic actions. The net effect of these two phenomena is an apparent cancellation of age vs. TRH dose effects denoted by a lack of significant F-ratios for interactions between the two factors.

Age-related effects for anesthetic activities have been noted by others: Streicher and Garbus (1955) observed that the duration of hexobarbital narcosis in male rats is prolonged with increasing age, from two months to six months

and from six months to 18 months. Buchsbaum, M., and Buchsbaum, R. (1962) also noted prolonged durations of diethyl ether anesthesia in mice with maturation from 0.5 months to 1-8 months, and from the latter stage to 14-20 months. Because the anesthesia durations were so rapid (lasting 3-4 min. only) and variable, it is difficult to extrapolate the data from the two month age group to the five month age group. However, the trend is also in the same direction of prolonged narcosis durations with early development. Further, it is even more difficult to extrapolate backwards from later development stages to early ones. Nonetheless, it is interesting to note that 12 month old male mice require twice the duration of six month olds to regain the righting reflex following i.p. ethanol injections, although brain content of the drug was similar for both age groups during loss and regain of the reflex.

It is difficult to state which intervening variables are responsible for this age-related difference. However, there are several important ones which could be implicated. The histological, histochemical and neurochemical findings reported by Samorajski, Rolsten, and Ordy (1971) suggest that the morphological and functional development of the mouse CNS and endocrine organs are still incomplete at three months of age. The brain, pituitary, and adrenal gland weights of C56BL/10 male mice significantly increase from three months to nine months, and later from nine months to 28 months of age. Mouse brain content and turnover of

neurotransmitters significantly increase during this period. In addition, pituitary and adrenal gland weights and also body weights significantly increase with age. Folch-Pi (1955) reported that between 75 and 180 days post partum, there are marked increases in whole mouse brain content of protein, lipids, proteolipids, cerebrosides, and cholesterol. Thus, it is clear that the brain, endocrine system, and soma of both two month old and three month old mice are still undergoing development of structure-function changes. In addition, increasing lipid and cholesterol content and body weight during development would entail increased barbiturate depot storage, and hence increasing narcosis durations. This viewpoint is supported by the analysis of variance results of the three month old mice data from Experiment 2. The analysis indicates that the barbiturate dose level was the most potent factor for this age group.

Toxicity. In all experiments, following co-administrations of TRH and pentobarbital to CF-1_S mice, all animals displayed a distinct sequelae which may be related to toxic and/or lethal drug interaction effects, effects predicted by Hypothesis 2. This sequelae has been described by a number of other workers (Breese et al., 1974b, 1975; Cohn et al., 1976; Crowley & Hydinger, 1976, 1977; Holaday et al., 1978; Prange et al., 1978b). The animals appeared to be marginally asleep, although they also displayed complete loss of both righting reflexes as well as purposeful movement. The animals displayed frequent stereotypic scratching

and grooming, shivering, shaking, severe myoclonic-tonic convulsions, and/or a dystonic (musculorum deformans) pattern with flaccid axial and limb musculature slowly writhing, twisting, and twitching. A fine, intense, extremely rapid tremor of both forepaws, and a stiff Straube-like arched tail was also observed in many animals. Twenty-two percent of mice receiving varying dose combinations of TRH plus pentobarbital died, while only 9% of mice receiving pentoparbital alone (50 mg/Kg or 70 mg/Kg, i.p.) plus saline (i.vt.) died. No animals died from either 10 μg or 20 μg TRH (i.vt.) plus saline (i.p.). Thus, there appear to be toxic interactions between the two drugs, effects which are either non-existent or are rarely observed when either drug is administered separately (in combination with saline). All animals that died from the varying drug combinations also displayed complete decerebrate rigidity, and the rigidity was found to be eliminated by cervical dislocation. Breese et al. (1975) found a systematic dose-dependent lethal effect for TRH-pentobarbital administrations, results which support the present findings. Although a 2 x 2 χ^2 test (1 df) revealed no significant differences in the lethal rate between drug treatment conditions, the highest drug-induced death rate (29%) was found for the 20 μg TRH plus 70 mg pentobarbital/Kg combination, followed by the death rate (21%) for 10 μg TRH plus 70 mg pentobarbital /Kg. Therefore, it is possible that the lack of dose-response effects observed for TRH's analeptic actions in

three month old mice could in part be explained as a masking of the analeptic effect by secondary toxic effects. The fact that these lethal effects were observed only in three month old mice (Experiment 2), but not in any of the two month old mice (Experiment 3), implicates an unanticipated critical stage in the development of the CF-1_S mouse with regard to TRH-barbiturate interactions. This is further supported by the fact that the two month old mice did display dose-related activities to TRH's analeptic actions, a finding in line with Hypothesis 1.

The symptoms described above may have significance in the localization of neurophysiological sites for non-specific TRH-pentobarbital interactions, i.e., actions unrelated to TRH's analeptic effects. The patterns described here are often cited both clinically (Chusid, 1976; Hollister, 1973) as well as experimentally (Iversen, S. D., & Iversen, L. L., 1975a, 1975b; Ungerstedt, 1973, 1974a) as being symptomatic of an extrapyramidal involvement in a Parkinson-like syndrome. While abnormally low dopamine levels in the basal ganglia usually have been identified as the biochemical basis for this syndrome, high ACh concentrations have also been maximally localized here and have been shown to have a strong modulatory role in the striato-thalamo-cortical Loop (Phillis, 1970; Shute & Lewis, 1967; Ungerstedt, 1974a; Valzelli, 1973). Most significant are the findings that high ACh concentrations in the corpus striatum have been shown to induce catalepsy and tremor;

these symptoms are ameliorated by anticholinergics; L-Dopa, a dopamine precursor, does not readily alleviate the tremor; and dopamine-induced stereotypies are potentiated by anticholinergics (Hollister, 1973; Iversen, S. D., and Iversen, L. L., 1975b; Ungerstedt, 1973, 1974b). In addition, Bloom, Costa, and Salmoiraghi (1965) found that barbiturates depress ACh-induced excitations of neuronal activities in the caudate nucleus. Trabucchi, Cheney, Racagni, and Costa (1975) reported that pentobarbital significantly decreases striatal ACh levels without altering transmitter turnover rates.

Neostriatum activities may be altered by TRH inductions. In this regard, Crowley and Hydinger (1976) reported that several monkeys receiving parenteral (i.m.) TRH injections displayed dose-related stereotypic behavioral patterns. TRH also evoked self-scratching behaviors which these investigators surmised to be a peptide-induced pruritic response. On the other hand, both of these behavioral patterns have often been implicated as extrapyramidal motor responses which are mediated by the dopaminergic nigrostriatal system (Ungerstedt, 1973, 1974b). A possible TRH influence upon dopaminergic activities is supported by the findings of Huidobro-Toro, Scotti de Carolis, and Longo (1974) who reported that TRH potentiates L-dopa plus pargyline (a monomine oxidase inhibitor; MAOI) induced stereotypic behavioral patterns and excessive agitated activities in a dose-related and time-related manner. Without pargyline pretreatments,

TRH was ineffective in this paradigm.

As stated here, this TRH-related dopaminergic activity, however, is probably non-specific to the TRH analeptic effect since: (a) TRH evokes DA release from the nucleus accumbens but not the caudate nucleus (Heal & Green, 1979), (b) Breese et al. (1975) reported that TRH does not affect brain content of DA or NE, nor does it affect brain content of monoamines in pentobarbital treated mice receiving pargyline pretreatments, and (c) chronic TRH administrations do not influence tyrosine hydroxylase (Nemeroff et al., 1977). Tyrosine hydroxylase is the enzyme which controls the rate-limiting step of catecholamine biosynthesis (Axelrod, 1974).

If TRH was administered before pentobarbital and allowed to dissociate away from its active CNS sites prior to the barbiturate administration, then one would predict an attenuation of both specific peptide analeptic actions as well as nonspecific drug interactions (Hypothesis 3). This was the purpose of comparing a 45 min. IID with a 1 min. IID in this project: Breese et al. (1975) found that the longest tolerable IID for maintaining TRH antagonism of pentobarbital narcosis was 30 min. between the two parenteral administrations. In addition, Burt and Snyder (1975) demonstrated that TRH has a radioreceptor kinetic dissociation half-life of approximately 38 min. Also, Winokur et al. (1977) found that TRH is enzymatically degraded in tissue homogenates after 30 min. at 37 °C. Thus, an IID longer than 30-40 min. between TRH pretreatments and pentobarbital

treatments should result in reduced peptide actions as predicted by Hypothesis 3. In support of this hypothesis, the results of Experiment 3 show that TRH was dose-related in the analeptic effect when a 1 min. IID was utilized; however, this dose-response relationship was disrupted when the 45 min. IID was utilized.

On the other hand, two month old mice did not display dose-related lethal effects (Experiment 3) while three month olds did (Experiment 2). Therefore, an attenuation of drug interactions in the younger mice would not be expected to facilitate the TRH analeptic effect in these animals. Instead, for this group, the longer IID actually attenuated TRH's efficacy. The analysis of variance and Scheffé t-test results further indicated that there were significant TRH dose-IID interaction effects only between 20 μg TRH and 0 μg TRH. Thus, the data indicate that the direct analeptic actions of the higher TRH dose was most sensitive to temporal (IID) influences. However, since the effects of a 45 min. IID were never tested in three month old mice, it is not possible to state whether this longer IID aids in the attenuation of non-specific toxic drug interactions.

Locomotor activity. Crowley and Hydinger (1976) reported that low doses of TRH injected (i.m.) into unanesthetized monkeys initially has no influence upon motor activities, and only slightly reduces these activities at 8-12 hours later. Higher doses of the peptide significantly reduce motor activities at all time periods. Crowley and

Hydinger (1977) also found that TRH administrations (i.m.) to monkeys significantly antagonize pentobarbital-induced depressions of motor activities. From these results, these investigators therefore concluded that TRH is not a motor stimulant (like amphetamines) but, rather, a central analeptic. Breese et al. (1975) also reported that TRH antagonizes the motor attenuation effects in mice of a number of central depressants, including phenobarbital. Breese et al. (1974a) reported that while TRH pretreatments (i.p.) significantly reduced ethanol-induced narcosis in mice, amphetamine pretreatments significantly enhanced it. They also administered the two drugs to unanesthetized animals and found that the mild increases in spontaneous locomotor activities of TRH treated mice over a 30 minute period were markedly below the activities elicited by amphetamine. These investigators concluded that the two drugs do not share a common mechanism of action.

The findings of the present study are also in line with the results of these investigators. In Experiment 3, the locomotor activities of mice receiving varying dose combinations of TRH (i.vt.) plus pentobarbital (i.p.) displayed: (a) significantly reduced, and never increased, locomotor activities for TRH pretreatments (compared to saline pretreatments) when observed for a 5 minute period following subsequent barbiturate treatments, and (b) no significant changes in locomotor activity for TRH pretreatments vs. saline pretreatments when observed before pentobarbital

treatments were administered. These findings support the viewpoint that TRH acts as an analeptic and not as a stimulant, i.e., TRH is a pharmacological antagonist of barbiturates, not a physiological one. Also, the findings indicate that TRH may have nonspecific motor depressant activities which partially mask the analeptic actions of this peptide. This viewpoint is supported by Crowley and Hydinger's (1976) report that higher doses of TRH (i.m.) produce grossly reduced motor activities (and sometimes symptoms of illness) in unanesthetized monkeys for 30 minutes after their administrations. This depressant action is then attenuated at the end of one-hour post-administration.

The analysis of variance results indicated that there were significant differences in TRH-induced alterations of locomotor activity between the two IIDs. The results showed that the 45 min. IID resulted in a higher activity level than the 1 min. Thus, as an extension of Hypothesis 3, it can be stated that the initial motor depressant interactions between the peptide and barbiturate were markedly attenuated when the two drug administrations were separated by a long IID.

Spare receptor effects. It was predicted that there would be a "ceiling effect" in the TRH analeptic effect, i.e., higher doses of the peptide would not augment the effect. This prediction for central TRH activities was based upon the spare receptor theory (Hypothesis 2) proposed for TRH's adenohypophyseal activities (Grant et al., 1973;

Vale et al., 1973; Vale & Rivier, 1975): This theory, as applied to TRH's central actions, states that only a small percentage of central TRH receptors needs to be occupied in order to obtain a full analeptic effect. Hence, beyond the threshold receptor occupation level, increasing peptide concentrations do not augment the analeptic effect.

On the other hand, if a spare receptor phenomenon did occur, the increasing IIDs should allow higher TRH doses to maintain efficacy, while lower dose effects would be attenuated due to enzyme degradation or kinetic dissociation (Hypothesis 3). Unfortunately, prior to the findings of this dissertation, the influence of the age factor upon TRH's analeptic effect was unknown. Therefore, although the present study indicated that three month old mice did not show dose-related effects to TRH's analeptic actions while two month olds did, the IID variable manipulation was tested only in the younger mice. These younger animals showed dose-response effects to the 1 min. IID, but attenuated responses to the longer 45 min. IID. There was no pharmacological evidence to warrant invoking a spare receptor theory for TRH activities in the younger animals. While this was the case for the older group, it was never tested under conditions where temporal effects were manipulated.

Cooperativity phenomena. The dissociation of TRH away from its pituitary receptor displays negative cooperativity phenomena during radioreceptor kinetics (Vale & Rivier, 1975). Burt and Snyder (1975) reported that a Scatchard

Analysis of their rat brain [^{3}H]-TRH radioreceptor data indicated the existence of two distinct classes of binding sites--a high-affinity and a low-affinity class. On the other hand, De Meyts (1976) proposed that a negative cooperativity theory is a better model for receptor data than the "multiple class" theory because: (a) it has no energy requirement, and (b) it only requires a single species of protein undergoing conformational changes.

Therefore, apropos to De Meyt's theory, and in line with the TRH kinetic findings of both Burt and Snyder, and also Vale and Rivier, a prediction of the present project was that negative cooperativity phenomena were expected for all TRH doses (Hypothesis 2). In essence, TRH was expected to display facilitated dissociation kinetics away from the peptide-receptor complex.

This facilitated dissociation was also expected to be observed on a behavioral level as an attenuation of the dose-response effects in the TRH analeptic effect. Longer IIDs were predicted to have a greater attenuating effect upon the analeptic actions of lower TRH doses because these lower doses would display facilitated receptor dissociations over time (Hypothesis 3). The higher TRH doses were also expected to display facilitated dissociations over the longer IIDs. However, the toxic and/or spare receptor phenomena displayed by the higher doses at short IIDs would now be reduced at the longer IIDs. Therefore, the relatively higher TRH concentrations which would remain at the receptor over this

longer IID period for the high TRH dose administrations would now be expected to be more effective as analeptics. In contrast, the lower dose TRH would not be greatly reduced below threshold concentrations at the receptor, and would therefore be less effective as an analeptic.

However, it should be noted that negative cooperativity was not observed in the radioreceptor assay. On the contrary, positive cooperativity phenomena were observed mainly at cerebral cortical postsynaptic TRH receptors (Experiments 7 and 8). Since this positive cooperativity entailed increased association of [^{3}H]-TRH in both the barbiturate competitive displacement radioreceptor assay as well as in the kinetic dissociation assay, this would suggest that this brain region is not a viable site of interaction for the TRH analeptic mechanism.

The other brain regions which displayed positive cooperativity in the kinetic dissociation assay--the limbic forebrain and the brain stem--also displayed specific high-affinity TRH binding and also specific high degrees of TRH receptor displacement by the barbiturates (Experiments 7 and 8). Therefore, cooperativity phenomena do not appear to influence the TRH analeptic mechanism. Hence, the different pharmacological results observed between Experiment 3 vs. Experiment 2, i.e., the presence vs. lack of TRH dose-response effects in the analeptic mechanism, must be explained on some other level besides cooperativity phenomena.

Diurnal variations. Despite Rubin et al's. (1974)

report of diurnal variations in pituitary release, the results of Experiment 2 suggest that there are no cyclical variations to the TRH analeptic effect. This finding was predicted in Hypothesis 4, because TRH's central analeptic actions have been shown to be independent of their traditionally studied pituitary-thyroid axis hormonal releasing actions (Breese et al., 1974b, 1975; Plotnikoff et al., 1972).

Biochemistry

The results of Experiment 4 indicated that there were no significant differences in regional and whole brain content of pentobarbital in the CF-1_S mouse brain at 1 hour post-treatments of 50 mg pentobarbital/Kg (i.p.) plus either 20 µg TRH (i.vt.) or physiological saline (i.vt.). All brain regions assayed under both conditions displayed barbiturate concentrations in the range of 18.0-20.2 µg pentobarbital/g original tissue. These biochemical results for both TRH-pretreated and saline-pretreated, pentobarbital-narcotized mice are in close agreement with the central pentobarbital assay reports by other workers for non-TRH pretreated, pentobarbital narcotized mice (Becker & Ho, 1977; Crane, Cornford, Braun, & Oldendorf, 1977; Lin & Sutherland, 1977; Yamamoto, Ho, & Loh, 1977). Becker and Ho (1977) reported no differences in regional and whole brain pentobarbital levels between barbiturate tolerant vs. non-tolerant mice. Their barbiturate values of 24.42 ($\pm$0.92) µg/g tissue at 50-70 min. following 60 mg Na pentobarbital/

Kg are very close to the present reported values.

Also, Lin and Sutherland (1977) reported that the regional brain distribution following administrations of [^{14}C]-barbital (i.p.) were fairly uniformly distributed regionally in the mouse brain at 30 min. following injections. Crane et al. (1977) also observed relatively uniform regional mouse brain levels of [^{14}C]-pentobarbital administered i.p. at most time periods studied, but most especially at 32 min. post-treatments. The hippocampus and brain stem regions, however, displayed slightly higher barbiturate levels. The brain showed a gradual uptake of barbiturate which maximized at 10 min. post-treatments, and remained at approximately a 100% mean body distribution level at 32 min.

Thus, it appears that the CNS levels of barbiturate are fairly uniform in both TRH-pretreated and saline-pretreated mice following 50 mg/Kg pentobarbital. Therefore, it can be concluded that: (a) since the TRH analeptic mechanism is not a function of altered brain barbiturate metabolism (predicted by Hypothesis 6), the TRH receptor remains a viable site to be considered for this mechanism (Hypothesis 5); (b) no new information can be gained about regional sites of TRH-barbiturate interactions from this biochemical assay, since brain levels of barbiturate are relatively uniform; and (c) Crane et al's. (1977) results might possibly implicate the hippocampus and brain stem as likely sites of interaction, since levels of barbiturate were slightly higher in these brain areas at 32 min. after

i.p. injections of barbiturate.

Ontogenesis of hepatic metabolism. The biochemical results of Experiment 6 indicate that there were significant age-related differences in the brain levels of pentobarbital between two month old and three month old male CF-1_S mice at 1.5 hr. following pentobarbital treatments in combination with either TRH or saline pretreatments. Regional barbiturate levels were uniform within age groups. TRH does not alter brain concentration levels of the barbiturate in comparison to physiological saline baseline levels.

Since TRH did not alter brain barbiturate concentrations, it can be surmised that the peptide is not affecting hepatic metabolism of the barbiturate. On the other hand, since there were significant age effects in brain concentrations of barbiturate, it can also be proposed that there are ontogenetic effects in the metabolism of barbiturate. Kato, Chiesara, and Frontino (1961) found that the *in vivo* metabolism of meprobamate is much more rapid in younger rats, and this metabolism decreases with age, from one month through six months post partum. In addition, younger rats display a greater induction capacity compared to older rats for increased meprobamate metabolism following pentobarbital administrations. Although it is difficult to directly extrapolate across the sexes, Stohs, Al-Turk, and Hassing (1980) found that in female Swiss-Webster mice, mixed function oxidase enzyme activities of hepatic and extrahepatic tissues undergo marked increases between one and six months

of age. Thus, the present results are in line with these latter findings.

Neurochemistry-Neurophysiology

Discrete brain receptor sites. The in vitro [^{3}H]-TRH radioreceptor assay data indicate that the limbic forebrain region of the adult male CF-1$_S$ mouse brain contains the highest concentrations of specific high-affinity TRH binding sites (Table 12). This region was shown to contain both presynaptic and postsynaptic TRH receptors with K_Ds in the range of 34.1-45.4 nM, and with concentrations in the range of 185.8-195.1 fmol/mg protein. These values are in close agreement with those reported by Burt and Taylor (1980) for the sheep brain nucleus accumbens, a subregion which was included in the limbic forebrain receptor assays of this dissertation. Burt and Taylor's radioreceptor assays also indicated that this brain area contained the highest concentration of TRH binding sites. Ogawa et al. (1981) gave a similar report for the monkey brain limbic forebrain. They also found that the cerebral cortex contained 75% of this concentration. Ogawa et al's. monkey brain results thus support the present mouse brain results, which indicate that the cerebral cortex contains only postsynaptic TRH receptors at 78% of the limbic forebrain concentration. In addition, Ogawa et al. reported finding two distinct classes of high-affinity TRH binding sites with K_Ds of 5.9 and 112 nM, respectively. These values are also matched by the 9.4-126.3 nM K_D range observed in this dissertation. The present

findings also indicated that the cerebral cortex contained the lowest concentration of TRH receptors which were also shown to possess the lowest TRH affinity; the brain stem region contained a high concentration (85% of the limbic forebrain level) of only presynaptic TRH receptors which were also shown to possess the highest affinity of all regional receptors studied.

The present mouse brain findings are also paralleled by Burt and Snyder's (1975) rat brain radioreceptor findings. They reported observing receptor concentrations of 150 and 190 fmol/mg protein for hippocampal and hypothalamic TRH receptors, respectively. They observed K_Ds of 36 and 52 nM for these two discrete receptors, respectively. Similar concentration and affinity values for the limbic forebrain were observed in the radioreceptor assays of this dissertation. In addition, Burt and Snyder's reports of 100 and 110 fmol/mg protein for mesencephalon and cerebral cortex receptors, respectively, are relatively similar to those of the present report. Thus, from the close agreement of the present radioreceptor findings with those of other workers, it can be assumed that the membrane receptor preparations of this dissertation were specific, regionally distinct, high-affinity TRH binding sites.

Competitive inhibition. In line with Hypothesis 5, which proposes that the TRH analeptic mechanism is directly mediated at specific TRH binding sites, the competitive displacement characteristics of the three barbiturate

analogues showed a relatively close correspondence to the binding site characteristics of discrete regional mouse brain TRH receptors (Table 12): All three analogues--phenobarbital, pentobarbital, and thiopental--displayed 100% inhibition of TRH at the brain stem presynaptic [^{3}H]-TRH radioreceptor. In conjunction with this finding, these receptors were found to have the highest TRH affinity, and the specific TRH binding to these receptors was 91% of the total in the radioassay. The next highest competitive displacement occurred at limbic forebrain postsynaptic [^{3}H]-TRH radioreceptors. At these sites, phenobarbital showed 96% TRH inhibition, and the other two analogues showed 61-63%. These receptors displayed the next highest TRH affinity, and also displayed 75% specific TRH binding of the total in the radioassay. Likewise, the limbic presynaptic radioreceptors which had the third highest TRH affinities and specific binding, also displayed the third highest competitive inhibitions by the barbiturates. The binding characteristics of these presynaptic receptors were similar to those of the postsynaptic receptors within this same brain area.

The postsynaptic receptors of the cerebral cortex which had the lowest TRH affinity, the lowest TRH binding site concentration, and the lowest specific binding, also displayed profound positive cooperative phenomena in the barbiturate competition: All three analogues similarly augmented, rather than inhibited, TRH binding at these radioreceptors by as much as 225-243%. This would indicate that

barbiturates facilitate retention of TRH binding to its specific cortical receptors. This finding parallels the kinetic radioreceptor data which also displayed positive cooperativity. In that latter assay, TRH increasingly associated, rather than dissociated, to its receptor by 24% over a 45 minute period. These latter findings do no, however, imply that a barbiturate-enhanced TRH binding indicates enhanced TRH analeptic actions. These receptors have a 56% nonspecific binding capacity, and thus the barbiturates may be retaining the peptide at these nonspecific sites without altering its intrinsic activity.

On the other hand, both presynaptic and postsynaptic limbic forebrain TRH receptors displayed high barbiturate competitive inhibitions in line with the high TRH affinity, specific binding, and binding site concentrations. While there was a great deal of positive cooperative phenomena in the respective radioreceptor kinetics for this region, these two receptor types did display TRH dissociations during the assay period. Thus, one could conclude that the TRH analeptic mechanism is mediated predominantly at limbic forebrain TRH receptors, a finding in close correspondence with Hypothesis 7. It should be added that the brain stem presynaptic receptors, which had the highest specific TRH affinity and binding and barbiturate competitions, also displayed positive cooperativity in the kinetics, but not competitive displacement, assay. In this case, as opposed to that for the cerebral cortex, the matching data strongly

implicate this brain area as a second likely site for TRH's analeptic actions.

The neuroanatomical substrate for the analeptic mechanism. Thus, the picture created by the radioreceptor data is one of brain stem presynaptic TRH receptor-containing axon terminals sending fiber projections and/or collaterals to presynaptic TRH receptors of the diencephalic component of the limbic forebrain. These presynaptic receptors then immediately innervate and synapse with diencephalic and telencephalic TRH postsynaptic receptors of this same brain system. Finally, the limbic forebrain postsynaptic receptors project more rostrally to postsynaptic TRH binding sites.

The radioreceptor data further indicate that the barbiturates are capable of competing with the peptide for many of these TRH binding sites (supporting Hypotheses 5 and 7). In line with Hypothesis 8, the in vivo radiochemical data of Experiment 8 (Table 13) also strongly supports this viewpoint: [^{3}H]-TRH redistributes after central (i.vt.) infusions to a number of discrete nuclei of the limbic forebrain--predominantly within the septal-hippocampal system, and the hypothalamus. Within this region, the tritiated peptide is shown to be particulate-bound, a finding which supports a receptor localization.

These findings are also supported by the RIA-subcellular fractionation studies of Winokur et al. (1977). They utilized both hypothalamic and extrahypothalamic rat brain homogenates. Their results indicated that the highest TRH

concentrations were in the crude mitochondrial fractions. Further isolation by density gradient centrifugation results in synaptosomal (M_2) fraction localization. In vitro exposure of these M_2 fractions to osmotic shock releases TRH from synaptic vesicles; and exogenous, but not endogenous, TRH added to brain homogenates at 37 °C is rapidly destroyed within 30-40 minutes (an effect greatly attenuated at 0°C and in methanol extracts). This latter finding is highly suggestive of protective membrane-bound storage mechanisms.

The in vivo radiochemical data for the pentobarbital-treated mice also supported the in vitro [^{3}H]-TRH radioreceptor findings of effective barbiturate competitive displacement in this latter assay (Hypotheses 7 and 8). At 1 hour post-treatments, pentobarbital effectively displaced the in vivo diffused [^{3}H]-TRH away from its predominant particulate-bound limbic forebrain localization to soluble cerebral cortex sites: septal-hippocampal-containing tritiation was reduced by barbiturate displacement from its saline baseline level of 38% of total brain levels to 18%, and pellet levels were reduced from 23% of the total to 6%; hypothalamic baseline-tritiation was reduced by barbiturate displacement from 21% of total brain levels to 9%, and pellet levels were reduced from 31% of the total to 13%. Prior to barbiturate treatments, the cerebral cortex possessed baseline levels of only 3% of total brain tritiation, 0% of total pellet, and only 4% of total soluble. After the tritiation was displaced away from limbic forebrain particulate-

bound loci by the barbiturate, the cortex suddenly possessed 19% of total brain tritiation, 11% of total pellet, and 21% of total soluble. These results match the radioreceptor results which also indicated that pentobarbital and other barbiturate analogues were effective competitive displacers of [^{3}H]-TRH away from limbic forebrain specific high-affinity presynaptic and postsynaptic TRH binding sites towards cortical postsynaptic receptors. These latter receptors display profound positive cooperative effects to both TRH alone and to [^{3}H]-TRH in the presence of the barbiturate analogues.

In addition, the radiochemical results indicated that there was a very low level of diffusible tritiation in the brain stem, both during saline baseline (5% of whole brain) and during barbiturate treatment (11% of whole brain) conditions. The *in vitro* radioreceptor results indicated a high concentration of specific high-affinity presynaptic TRH binding sites in this region. These receptors were shown to display a high degree of effective competitive displacement of [^{3}H]-TRH by each of the barbiturate analogues. However, the fact that this region received such low *in vivo* peptide levels indicates that this CNS region is less involved in TRH analeptic actions than the limbic forebrain region. Also, pellet-bound tritiation was substantially increased in this region (from a baseline of 13% to 18% of the whole brain level) following the *in vivo* barbiturate displacement of pellet-bound [^{3}H]-TRH from the limbic forebrain. This

indicates that there were positive cooperative effects in the in vivo barbiturate condition. The brain stem receptors did display positive cooperativity to TRH alone in the radioreceptor assay. Thus, the composite picture presented by the two types of assays indicates that while the brain stem is capable of an in vitro competitive displacement of TRH by barbiturates, during the in vivo barbiturate condition there is a relatively limited-capacity, yet high-affinity pellet-bound retention (positive cooperativity) of the peptide at the receptor. This pellet binding occurs in the brain stem in conjunction with peptide displacement.

The viability of utilizing brain synaptic membrane receptor preparations as representative sites of interaction between TRH and barbiturates. Competitive radioreceptor TRH binding studies utilizing synthetic tritiated TRH in pituitary tissues (mouse thyrotropic and rat somototropic/prolactotropic tumor, as well as normal bovine and rat membrane preparations) reveal high specificity of binding: [^{3}H]-TRH binding is saturable; unlabelled TRH is capable of stoichiometric competition; and the majority of binding sites are localized in the plasma membrane subcellular fraction, which displays an approximate 40-fold increase in [^{3}H]-TRH binding when compared to the total adenohypophyseal homogenate (Burt & Snyder, 1975; Burt & Taylor, 1980; Grant et al., 1973; Labrie, Barden, Poirier, & De Lean, 1972; Labrie, De Lean, Lagrace, Drouin, Ferland, Beaulieu, & Morin, 1978; Poirier, Labrie, Barden, & Lemaire, 1972). In addition, a large

number of synthetic TRH structural analogues have been synthesized and studied in the pituitary (Burt & Snyder, 1975; Burt & Taylor, 1980; Grant et al., 1973; Vale et al., 1973; Vale & Rivier, 1975; Vale et al., 1977; Vale, Rivier, & Burgus, 1971). In general, the relative in vivo biological potency (percentage TSH release; percentage PRL release) of these analogues correlates fairly well with their in vitro radioreceptor [^{3}H]-TRH competitive binding capabilities (relative affinities) in pituitary membrane receptors. Potent receptor antagonists, mainly TRH analogues, capable of blocking TRH-stimulated release of TSH have been synthesized (Bowers, Sievertsson, Chang, J., Stewart, Castensson, Björkman, Chang, D., & Folkers, 1976; Lybeck, Leppäluoto, Virkkunen, Schafer, Carlsson, & Mulder, 1973; Sievertsson, Castensson, Andersson, Björkman, & Bowers, 1975).

However, it is important to note that the physiological functions of brain TRH receptors, i.e., analeptic activities, appear to be different from pituitary receptor functions, since: (a) in general, hypothalamic neuropeptides have been shown to have actions in the CNS independent of their pituitary-mediated endocrine activities (see General Implications); (b) more specifically, TRH's analeptic actions occur independent of pituitary activities (see TRH Antagonism of Barbiturate Narcosis); (c) brain vs. pituitary TRH binding sites display similar, yet distinct, recognition requirements for TRH structural analogues (see Specific

Saturable Binding); (d) the efficacy of TRH analogues in the mediation of analeptic actions can be dissociated from their competitive binding affinities in pituitary TRH receptor preparations (see Critical Issues); and (e) TRH may act as an extraendocrine central neuromodulator (see Evidence for TRH as a Neuromodulator). Thus, while brain and pituitary TRH receptors have similar in vitro affinities for the parent TRH molecule, their respective in vivo functions appear to be distinct from one another. It would therefore seem necessary to consider the general structure-function relationships of neuronal binding sites as a basis for further characterizing the possible underlying central receptor mechanism of the TRH analeptic effect (Hypothesis 5).

De Robertis (1971) isolated nerve-ending membranes and junctional complexes (synaptosomes) via cellular fractionation of regional brain tissues, including the cerebral cortex, basal ganglia, hypothalamus, and mesencephalic nuclei. These synaptosomes were shown to possess in vitro high-affinity binding characteristics for radiolabelled cholinergic and adrenergic blocking agents, 5-HT, His, and psychotropic drugs (lysergic acid diethylamide, chlorpromazine, and trifluprомazine). This indicates that central receptors are localized postsynaptically in the subsynaptic membrane. Subsequent organic extraction of these membrane components further localizes this high-affinity binding within proteolipids. In addition, physicochemical methods (such as

polarization of fluorescence and light scattering) provide evidence that the proteolipids of these receptors undergo conformational changes in the presence of various group-specific ligands (specific per receptor type). These ligand-induced conformational changes are accompanied by characteristic cooperative phenomena.

Cytochemical and morphological electron microscope analysis of the postsynaptic density (PSD) component of plasma membranes (derived from rat forebrain tissues) reveal that this isolated component exhibits the same structural and physiological functions as in intact tissue (Banker, Churchill, & Cotman, 1974; Cotman, Banker, Churchill, & Taylor, 1974; Cotman & Taylor, 1972). Quantitative analysis indicates that there is a 20-fold enrichment of PSDs after isolation from the synaptic junction-containing membrane fraction, that PSDs are then about 85% pure, and that they are highly adhesive. Proteolytic enzyme analysis as well as polyacrylamide gel electrophoresis indicate that PSDs consist mainly of polypeptides which are integral to their structure. The PSDs are found to be distinctive structural specializations associated with dendritic synapses and are essential to the mediation of synaptic transmission functions.

Hence, it can be surmised that the postsynaptic receptor membrane preparations utilized in the present project, which were isolated by the same procedures as above, should represent highly purified and specific high-affinity TRH

binding sites, i.e., the receptors should have displayed high affinities for both TRH-specific agonists and antagoists. It can be further postulated that both proteolipid conformational changes and cooperative phenomena would have been expected to occur following in vitro exogenous applications of neurochemical and pharmacological agents which are capable of specific binding and efficacy at these TRH binding sites. There would also be the likely possibility that the degree of specificity for regional TRH binding determined by the present radioreceptor assay would be much higher than the binding specificity values reported by other workers not utilizing the 20-fold purified receptor membrane preparations (e.g., Burt & Snyder, 1975; Burt & Taylor, 1980).

The purified presynaptic receptor membrane preparations of this project were isolated by the differential and density gradient centrifugation procedures of Essman, W. B., and Essman, S. G. (1977). These procedures have been modified from the procedures of Israel and Whittaker (1965) and Whittaker and Barker (1972), and would thus be expected to also be viable, highly specific, and functional TRH binding sites. This position is supported by the plethora of evidence implicating the presynaptic membrane as the neuronal regulatory site for biosynthesis, storage, release, and metabolism of neurotransmitters (Andén, Bedard, Fuxe, & Ungerstedt, 1972; Axelrod, 1974; Couteaux, 1974; De Robertis, 1971; Karczmar & Nishi, 1971; Simon & Kuhar, 1975; Snyder &

Coyle, 1969). In addition, Schaeffer et al. (1977) demonstrated in vitro potassium-stimulated, calcium-dependent endogenous release of TRH from rat hypothalamic and septal synaptosomes in transmitter-like fashion.

Neurotransmission influences. A large series of studies indicate that TRH-barbiturate interactions influence membrane excitability and transmitter release: Blaustein (1967) and Blaustein and Goldman (1966) demonstrated that the three barbiturate analogues utilized in the present radioreceptor study--phenobarbital, pentobarbital, and thiopental--are capable of binding in vitro as [^{14}C]-radiolabelled ligands to membrane phospholipids, phosphatidyl serine, and phosphatidyl ethanolamine. This binding was increased in the presence of calcium (Ca^{+2}) ions, and conversely, the barbiturates reciprocally induced dose-dependent increases in the binding of the cation. These investigators proffer the Davson-Danielli model of the nerve membrane as a lipid bilayer coated by protein on both its inner and outer surfaces, and with ion exchange phospholipids making up a significant proportion of the external lipid layer. Hence, on the basis of Ca^{+2} effects upon the electrophysiological properties of nerve fibers, these researchers proposed that the first step in the depolarization of a nerve may be the removal of Ca^{+2} from the site on the membrane to which it is bound. It was further proposed that the anionic head of the lipid-soluble barbiturates could serve to neutralize the cationic nitrogen moiety of the

phospholipid polar head after inserting itself between the phospholipid fatty acid chains. The barbiturates would thereby increase the tendency of divalent ions such as Ca^{+2} to bind to these same sites, which would then subsequently reduce the capacity of the phopholipids to change configuration and ion specificity under the influences of a changing electric field. Hence, the magnitude and rate of sodium (Na^{+}) and potassium (K^{+}) ion conductances would be increased in the nerve membrane, and this would then result in a consequent reduction in the action potential amplitude.

Many other studies support the viewpoint that barbiturates influence Ca^{+2} binding at the neuronal membrane phospholipid layer, and thereby alter ionic conductances, depress neurotransmission, and block receptor sensitivities as well as the ensuing behavioral concomitants (Blaustein, 1967; Blaustein & Goldman, 1966; Brink, 1954; Bloom et al., 1965; Crawford & Curtis, 1966; Galindo, 1969; Krnjevic & Phillis, 1963; Larrabee & Posternak, 1952; Shanes, 1958; Somjen, 1963; Thesleff, 1956). Lüttgau (1963) noted that exogenous bathings of frog semitendinous and ilofibularis muscles with Ca^{+2} ions evoked marked changes in the Ca^{+2}-dependent membrane potentials of the sodium-carrying system while also altering isometric contractions. In this regard, Thesleff (1956) found that exogenous bathings of frog end-plate membranes with pentobarbital caused a marked dose-dependent and reversible reduction of microiontophoretically applied ACh depolarizations. Haycock, Levy and Cotman

(1977) found that pentobarbital selectively inhibits the depolarization-induced and Ca^{+2}-dependent release of GABA from brain synaptosomes, and Barker and Ransom (1978) found that the barbiturate depresses postsynaptic GABA membrane excitability.

Electrophysiological and neurochemical studies also indicate that TRH is capable of altering spontaneous and excited neuronal transmission and also reversing barbiturate-induced changes: Yarbrough (1976, 1978) and Yarbrough and Singh (1978) discovered that microiontophoretic and parenteral TRH applications potentiate the muscarinic excitatory actions of ACh and carbamylcholine upon the somatosensory cortical neurons of the pentobarbital anesthetized cat. Trabucchi et al. (1975) found via radiometric gas chromatography analysis that parenteral pentobarbital administrations significantly increased ACh and choline content and significantly reduced ACh turnover in the mouse brain. In contrast with this effect, Malthe-Sørenssen et al. (1978) studied a number of neuropeptides and found that TRH was the only one capable of altering ACh turnover in the rat parietal cortex. Also, Schmidt (1977) found that while TRH had no effect in unanesthetized rats, the peptide antagonized both pentobarbital-induced decreases in Na^{+}-dependent, high-affinity choline uptake as well as the increased regional ACh levels of the rat brain. These TRH findings are important in light of the observations that barbiturates are capable of inducing depressions of neuronal choline-uptake

and ACh turnover concomitant with decreased neuronal activities and augmented transmitter reserve pools in the rat hippocampus, hypothalamus, and cerebral cortex (Atweh et al., 1975; Moroni, Malthe-Sørenssen, Cheney, & Costa, 1978; Simon et al., 1976; Simon & Kuhar, 1975). Similar to TRH actions, electrical stimulations of the septum reverse these barbiturate effects. These findings seem to fit under the larger framework of information in this area which implicates depressed neuronal activities concomitant with decreased transmitter turnover and release following central depressant pharmacological administrations, and the reversal of these effects by central stimulants, analeptics, and convulsants (Atweh et al., 1975; Elliott et al., 1950; Giarman & Pepeu, 1962; Malthe-Sørenssen et al., 1978; Phillis, 1968; Richter & Crossland, 1949; Tobias et al., 1946).

Many other studies also support the viewpoint that both the barbiturates and TRH alter neurotransmission: Richter and Jackson (1980) and Waller and Richter (1980) demonstrated that pentobarbital inhibits K^{+}-stimulated, Ca^{+2}-dependent release of ACh, Glu, aspartate, but not 5-HT, from rat midbrain tissue slices. Jessel and Richards (1977) found that pentobarbital depresses GABA release but not uptake from rabbit hippocampal tissue slices. However, they found that the barbiturate does not influence [^{3}H]-GABA binding to postsynaptic receptor membranes. Also, Ngai, Wang, and Spector (1980) replicated this latter finding in the rat brain by utilizing [^{3}H]-muscimol radioreceptor

assays. Sato, Austin, and Yai (1967) provided electrophysiological evidence that exogenously applied pentobarbital is capable of binding to ACh postsynaptic membrane receptors. The functional characteristics of this binding is witnessed as a marked dose-dependent and reversible augmentation of membrane depolarizations subsequent to profound increases in K^+ ion permeability, findings in line with Nernst equation predictions.

As already noted, Malthe-Sørenssen et al. (1978) reported that TRH increases ACh turnover in the rat parietal cortex. Heal and Green (1979) found that TRH releases DA from the nucleus accumbens. Green and Grahame-Smith (1974) found that TRH pretreatments potentiate the hyperactivity reactions of rats treated with tranylcypromine plus L-tryptophan, although the peptide does not directly alter brain concentrations of 5-HT or tryptophan. Keller et al. (1974) reported that TRH enhances NE turnover and [^{14}C]-tyrosine incorporation into [^{14}C]-NE of the rat brain without changing incorporation into [^{14}C]-DA. On the other hand, Breese et al. (1975) found that TRH had no influence on the incorporation of tritiated tyrosine into brain NE, nor did it alter brain content of NE, DA, or 5-HT of pentobarbital-treated mice. Schaeffer et al. (1977) found that only DA, but not NE, GABA, 5-HT, carbachol, and taurine, can induce transmitter-like <u>in vitro</u> release of TRH from hypothalamic synaptosomes. Warberg et al. (1977) replicated and further extended these findings of both spontaneous and

dopaminergic-induced TRH release.

Results by Renaud and Martin (1975) and Renaud et al. (1975, 1976) show that microiontophoretically applied TRH has marked depressant effects on spontaneous and glutamate-evoked unit discharge rates for small select neuronal populations of the ventromedial-arcuate hypothalamus and parietal cortex of the rat brain, and to a lesser degree cerebral cortex and brain stem neurons. Winokur and Beckman (1978) reported that there are some overlapping similarities in the sensitivities of select neuronal populations of the rat brain hypothalamus and septum to either TRH or NE applications. Dyer and Dyball (1974) also reported that TRH and luteinising hormone release factor (LRF) can selectively modulate the spontaneous neurotransmissions of independent discrete populations of hypothalamic neurons. As noted earlier, the findings of Yarbrough (1976, 1978) and Yarbrough and Singh (1978) indicated that TRH potentiates ACh responsitivities of cortical neurons.

To summarize, it appears that barbiturates are capable of altering neuronal membrane excitability via their binding to membrane phospholipid layers; this activity then induces altered cation binding to the membrane, which subsequently alters membrane conformational changes and ionic permeabilities. The net effect of these barbiturate actions are depression of neuronal transmission, and transmitter release, turnover, and uptake. TRH profoundly reverses all of these barbiturate actions at the receptor level--both

presynaptically and postsynaptically. The net effect is a TRH-induced increase in neuronal transmission levels in opposition to barbiturate-induced depressions. These latter findings strongly support Hypothesis 5, which proposes that the TRH analeptic mechanism is mediated at specific TRH receptors. In support of Hypotheses 7 and 8, respectively, the radioreceptor and radiochemical data indicate that these interactions are occurring predominantly within septal-hippocampal and hypothalamic neuronal populations of the mouse limbic forebrain; to a lesser degree in the brain stem; and to a questionable degree in the cerebral cortex. These TRH actions appear to be the basis for the analeptic mechanism.

It is very important to note, however, that Burt and Snyder (1975) and Burt and Taylor (1980) tested a large series of neurotransmitters and related pharmacological agents in rat and sheep brain-derived [^{3}H]-TRH radioreceptors. Their results showed that none of the classical transmitters and agents had any influence as competitive displacers of TRH at discrete specific central TRH binding sites. Also, as noted earlier, TRH may not alter catecholamine or 5-HT biosynthesis or storage levels in the rodent brain (Breese et al, 1975; Nemeroff et al., 1977). In addition, Bloom et al (1965) found that barbiturates do not affect activities of caudate units which were responsive to GABA and Glu applications.

Thus, it appears that TRH's analeptic actions may be

directly TRH receptor mediated, while other TRH, barbiturate, and TRH-barbiturate extra-TRH classical transmitter-related actions are probably secondary effects. In this regard, it can be noted that Breese et al. (1975) found that central administrations of both atropine and methyl atropine antagonized the TRH analeptic effect. On the other hand, neither peripheral administrations of methyl atropine (which has difficulty being transported across the blood brain barrier) nor central administrations of phentolamine (an adrenergic receptor antagonist) had any influence on this TRH effect. Thus, the TRH analeptic effect may possess an indirect cholinergic component. This position would certainly fit in with the generally accepted viewpoint noted earlier that cholinergic neuronal activities markedly fluctuate following endogenous and exogenous influences on organismic states of sleep-wakefulness and/or narcosis-arousal.

Neuroanatomical considerations. The classical electrophysiological studies of Dempsey and Morison (1942a, 1942b), Morison and Dempsey (1942), and Moruzzi and Magoun (1949) showed that repetitive electrical stimulation (at a frequency of 8-12/sec.) of the diffuse thalamic projection system can act as a cortical "pacemaker" in the recruiting of the cortical electroencephalogram (EEG) responses. In addition, these studies showed that high frequency electrical stimulations of the brain stem reticular formation lead to a block of the diffuse thalamic-induced recruiting response. Forbes and Morison (1939) found that these cortical

recruiting after-discharges--which they labelled the "Forbes Secondary Response (FSR)"--were associated with altered states of consciousness, i.e., high doses of barbiturate anesthesia blocked both the cortical FSR after-discharges to sensory stimuli while also blocking human subject awareness of these stimuli. The barbiturate-induced block of FSR after-discharges has been replicated many times (Brazier, 1955; Bremer, 1937; Chang, 1950; Swank & Watson, 1949). In addition, French, Verzeano, and Magoun (1953) discovered that pentobarbital alters the form of reticular response potentials, indicative of a barbiturate-induced suppression of neuronal activity within the diffuse activating projection system. Bradley and Key (1958) added that these barbiturate actions are dose-related since only high doses could suppress the cortical EEG and behavioral arousal thresholds of encéphale isolé cat preparations to both adequate and direct electrical reticular stimulations. Arduini, A. and Arduini, M. G. (1954) simultaneously recorded cortical (auditory Projection area) and reticular potentials from encéphale isolé cats receiving adequate (auditory) and direct reticular stimulations, respectively. They observed dose-related and reversible effects following barbiturate administrations: Lower doses of pentobarbital specifically suppressed only reticular responses of the cat to adequate stimuli, while increasing doses also suppressed cortical activities. They found that sodium cyanide, a known cellular respiratory blocker, rapidly attenuated specific

reticular responses; subconvulsive doses of metrazol, a central stimulant, augmented both reticular and cortical responses; strychnine selectively augmented reticular responses. Hence, these results further implicate the reticular formation as a primary site of action for both barbiturate anesthesia as well as pharmacological stimulations. Other classical ablation-pharmacological-electrophysiological-behavioral studies strongly implicate the ARAS component of the reticular formation in the mediation of altered states of consciousness during normal sleep-wakefulness as well as during exogenous stimulatory-inhibitory influences (Bradley, 1958; French & Magoun, 1952; Lindsley, Bowden & Magoun, 1949; Lindsely et al., 1950; Livingston, 1959; Moruzzi & Magoun, 1949). A cholinergic component of the ARAS has been anatomically identified (Lewis & Henderson, 1980; Shute & Lewis, 1963, 1967), with dorsal and ventral tegmental pathways projecting to tectal, thalamic, and basal forebrain regions, which then project more rostrally to the cerebral cortex and olfactory bulbs. Studies by Bradley (1958) and Phillis (1968) showed that both pentobarbital and atropine act within levels of the ARAS. These latter findings are in line with the previously cited studies which indicated that both barbiturate vs. stimulatory, analeptic, and convulsant pharmacological agents can alter CNS cholinergic levels--especially within the ARAS--in an antagonistic manner. In this regard, it should again be noted that Breese et al. (1975) were able to block TRH antagonism of

pentobarbital-induced narcosis by central administrations of atropine, a classically utilized ACh receptor antagonist. The studies of Yarbrough (1976, 1978) and Yarbrough and Singh (1978) indicate that microiontophoretically-applied TRH facilitates cortical neuronal responses to ACh in pentobarbital anesthetized rats. Thus, it appears that TRH's analeptic mechanism may indirectly influence cholinergic components (Burt & Snyder, 1975; Burt & Taylor, 1980, reported that atropine and other cholinergic agents did not compete at specific TRH receptors) of a diffuse arousal projection system, a system traditionally identified with altered states of consciousness, including pharmacological anesthesia and arousal.

In addition, as noted earlier, the studies by Green (1960) and Green and Arduini (1954) indicated that asynchronous hippocampal theta (θ) activity could be generated by adequate stimuli which also produce behavioral and cortical electrographic arousal. Pentobarbital profoundly attenuates neuronal activities within the hippocampal, hypothalamic, and cortical components of Papez' limbic circuit. These effects are accompanied by reduced neuronal membrane uptake of choline, reduced ACh turnover, and increased transmitter pools (Atweh et al., 1975; Simon et al., 1976; Simon & Kuhar, 1975). Either direct electrical MRF or septal stimulations, catecholaminergic agents, or microinjections of TRH into rat brain regions produce a profound reversal of the neurochemical, electrophysiological, and

behavioral concomitants of the barbiturate-induced depressant activities (Atweh et al., 1975; Hernández-Péon, & Chávez-Ibarra, 1963; Hernández-Péon, Chávez-Ibarra, Morgane, & Timo-Iaria, 1963; Kalivas & Horita, 1979, 1980). The septal region was the brain area most implicated in these effects. Other moderate-high sensitive regions were found in both the forebrain and brain stem components of the limbic system, which included: The nucleus accumbens, the medial hypothalamic-preoptic area, medial thalamus, dorsal hippocampus and mesencephalic reticular formation-interpeduncular nucleus-periventricular gray pathway. These findings are also supported by Kramis and Vanderwolf's (1980) report that electrographic hippocampal θ responses could be evoked by electrical stimulation of specific septal, hypothalamic, and brain stem sites. They were able to distinguish between atropine-anesthesia (urethane) sensitive vs. non-sensitive "frequency driving sites". The drug sensitive sites included the medial septum, diagonal band of Broca, the medial forebrain bundle, the dorsomedial hypothalamic nucleus, the central gray, some substantia nigra sites, and some brain stem reticular formation sites.

Thus, in support of Hypotheses 5, 7, and 8, both the former and latter results strongly implicate the septal-hippocampus and hypothalamic components of the limbic forebrain, as well as brain stem limbic sites as the neuroanatomic substrate for the TRH analeptic mechanism. This position is further supported by the present <u>in vitro</u> [^{3}H]-TRH

radioreceptor and *in vivo* radiochemical findings. The present findings also match the microinjection results of Kalivas and Horita (1979, 1980); the radioreceptor results of Burt and Snyder (1975), Burt and Taylor (1980), Ogawa et al. (1981); and the results of a large series of RIA and histochemical studies which have localized the regional distribution of TRH within these same storage and axon terminal sites (Brownstein et al., 1974; Hökfelt et al., 1974, 1975; Kubek et al., 1979; Leppäluoto et al., 1978; Spindel & Wurtman, 1980; Youngblood et al., 1978).

Summary and Conclusion

The findings of this dissertation indicate that TRH is a very potent central analeptic in the *in vivo* antagonism of barbiturate-induced narcosis of the adult male CF-1_S mouse. The results of the *in vitro* radioreceptor assays indicate that TRH has specific high-affinity binding sites in the mouse brain, which are discretely distributed and which are distinguishable as either presynaptic or postsynaptic. These results also indicate that the TRH analeptic mechanism is mediated at several of these brain sites since barbiturate analogues are capable of effectively displacing the peptide from its specific brain receptors. The *in vivo* [^{3}H]-TRH radiochemical assay data are in close agreement with the radioreceptor findings. The CNS region most implicated as the neuroanatomical substrate for the analeptic mechanism is the limbic forebrain, with the septal-hippocampus system and hypothalamus displaying high concentrations

of specific TRH binding sites as well as high degrees of competitive displacement by barbiturates. The brain stem, a rather widespread brain area containing the interpenduncular nucleus, mesencephalic reticular formation, and perventricular gray, also contains a high concentration of specific TRH receptors. This region displays the highest TRH affinity of all regional receptors studied, and it also displays the highest levels of barbiturate competition in the radioreceptor assay. The cerebral cortex, however, is less strongly implicated in the analeptic mechanism since it displays high positive cooperativity phenomena in the competitive displacement of [^{3}H]-TRH by barbiturates at the TRH receptor. In addition, its specific receptor binding is only 44% of the total, as compared to the limbic forebrain and brain stem receptors which show 75% and 91% specific binding. The cortex also displays positive cooperativity in the binding of [^{3}H]-TRH to its receptors.

The behavioral pharmacology data suggest that central administrations of the peptide are capable of showing dose-response relationships in the central effect, but age-related and temporal factors can alter these relationships. The order of drug administrations does not seem to be a crucial factor because: (a) TRH is effective in the antagonism of barbiturate narcosis whether it is administered prior to or following barbiturate administrations; (b) changing the order of interinjection durations of treatments in a repeated measures paradigm does not appear to alter the

efficacy of the peptide activity. Cyclical diurnal variations were not found to be present in the TRH effect. This was not a surprising finding in light of a number of lines of evidence suggesting that TRH's central actions are independent of fluctuating pituitary functions. Some evidence is provided to suggest that TRH displays toxicity in its interactions with barbiturates.

The general findings are in close correspondence with a plethora of studies which implicate both TRH and barbiturate actions as being localized at distinct CNS regions innervated by the forebrain and brain stem components of Papez' limbic pathway. Evidence has been provided to suggest that TRH and barbiturates act in a reciprocal manner to alter neuronal activities within these distinct regions. In conclusion, it would seem crucial to synthesize and characterize newer TRH structural analogues in order to effect a treatment regime for the reversal of barbiturate overdose without inducing other forms of toxicity.

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