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Early Postnatal Administration of 5,7-Dihydroxytryptamine: Effects on Substance P and Thyrotropin-Releasing Hormone Neurons and Terminals in Rat Brain

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Abstract

Substance P, thyrotropin-releasing hormone (TRH) and serotonin are putative neurotransmitters which have been proposed to coexist in some brain neurons Our previous immunocytochemical and biochemical studies have demonstrated that 85–100% of all serotonin neurons are destroyed following neonatal 5,7-dihydroxtryptamine (5,7-DHT) treatment In this study, we have determined the effect of neonatal 5,7-DHT and desipramine (DMI) treatment on the biochemical content and immunocytochemical localization of substance P and TRH throughout the brain Interestingly, we have observed that virtually all substance P- and TRH-immunoreactive cells in the ventral pons-medulla are destroyed by the neurotoxin. However, peptide-containing neurons in other regions were not affected Additionally, we measured the peptide content and found that TRH is significantly decreased in the spinal cord (−50%) and pons-medulla (−20%), but not in other brain regions Substance P content was not significantly altered in any region, even after a greater than 90% reduction of serotonin These data indicate that the co-localized substance P and TRH forms a small proportion of the total peptide in brain

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Keywords

substance P; thyrotropin-releasing hormone (TRH); serotonin; 5,7-dihydroxytryptamine; neurotoxin; co-localization

Introduction

Substance P and TRH are neuroactive peptides which have been proposed to co-exist with serotonin in some neurons of the rat brain[8,9,16]. Johannson et al [20] have shown that serotonin immunoreactivity is present in medullary neurons which also contain TRH and/or substance P. This quantitative immunocytochemical study has indicated that relationships between these compounds are complex, as demonstrated by the variable populations of hindbrain serotonergic neurons which contain none, one or both peptides Another approach to demonstrate co-localization existence consists of the treatment of adult rats with the selective serotonin neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT) and measurement of changes in TRH or SP content by radioimmunoassay[5,7 12,24,30]. These biochemical studies have generally indicated that substance P and TRH content in the ventral spinal cord declines concomitantly with a decrease in serotonin content.

In recent reports[6,35] we have demonstrated that 5,7-DHT treatment of neonatal rats leads to permanent destruction of virtually the entire serotonergic neuronal population. Intracisternal injection of 5,7-DHT into 3-day-old rats leads to the rapid degeneration of 80–98% of serotonergic neurons throughout the brain Pretreatment with desipramine or pargyline effectively protects catecholamine neurons from toxic effects of 5,7-DHT In contrast, previous paradigms for 5,7-DHT-induced lesion of serotonergic neurons have been only partially effective, since treatment of adult rats leads to pruning of distal terminals without any apparent proximal damage and loss of cell bodies In this investigation, we applied our technique for the total destruction of the serotonergic system to determine whether or not cells containing the putative co-localized peptides TRH and SP are lesioned concurrently and further, to assess the relative contribution of the co-localized peptide to the total content throughout the brain

Materials and Methods

Animals

Three-day-old Sprague–Dawley rats (Charles River Breeders, Willmington, MA) received an intracisternal injection of 50 μg 5,7-DHT, 1 h after desipramine (DMI; 20 mg/kg i.p.). Animals were treated with capsaicin (50 mg/kg) on day 5 ([ref. 19]). Control rats received DMI alone. Some animals sacrificed for immunocytochemistry were pretreated with cholchicine (100 μg, intraventricularly) 24 h prior to perfusion.

Biochemistry

Substance P content was determined by a modification of the radioimmunoassay as described by Cuello et al.[10], using a monoclonal substance P antibody (clone NCI/34; Seralabs, Crawley, U.K.) in a non-equilibrium assay. Briefly, tissues were homogenized in 5% trichloroacetic acid, 5 mM ascorbic acid and centrifuged at 24,000 *g* for 10 min. An aliquot of the supernatant fraction was extracted 3 times with water-saturated ether to remove the trichloroacetic acid. A 100 μl aliquot was preincubated with 100 μl antibody (1:4000) for 3 h and then 10,000 dpm of [^{125}I]Tyr^{8}-substance P was added. After the assay mixture was incubated overnight with shaking at 4 °C, the antibody bound substance P was precipitated with an equal volume of 100% saturated ammonium sulfate. The precipitate was

rinsed with 50% saturated ammonium sulfate and was counted The [^{125}I]Tyr^{8}-substance P was synthesized by the procedure of Hunter and Greenwood[18] and purified on a 20 cm Sephadex G-25 column equilibrated with 0.1% bovine serum albumin.

TRH content was determined in a similar radioimmunoassay, using an aliquot from the ether extracted supernatant. The TRH antibody was prepared in rabbits immunized with a TRH-BSA conjugate formed with bis-diazotised benzidine[1]. A 100 μl sample was combined with equal volumes of TRH antisera (1:1000) and 10,000 dpm of [^{125}I]TRH. After incubating overnight, the antisera-bound TRH was precipitated with saturated ammonium sulfate, rinsed and counted on a gamma counter.

Serotonin was determined in the initial trichloroacetic acid supernatant by a reverse-phase high-performance liquid chromatography (HPLC) method which has been described previously[22]. Protein concentrations were determined by the method of Lowry et al.[25], using bovine serum albumin as a standard.

Immunocytochemistry

Animals were sacrificed with chloral hydrate (40 mg/kg, i.p.) and prepared for serotonin and substance P immunostaining by transcardial perfusion with 4% paraformaldehyde, pH 6.8, followed immediately by 4% paraformaldehyde, pH 10.5 ([ref. 3]). The brain was processed through ethanol and toluene for routine embedding in Paraplast plus (Fisher). Following rehydration, serial 10 μm coronal sections were treated with trypsin (1.2 mg/ml PBS, Boehringer Mannheim) for 10 min, rinsed extensively and incubated for 48 h at 4 °C with a monoclonal substance P antibody (1:1000; Seralabs, Crawley, U.K.) or a serotonin antisera (1:2000). The primary serotonin antiserum and substance P antibody were localized using a modified 'ABC' technique which has been described previously[17-34]. Since the monoclonal substance P antibody was a rat IgG, we used biotinylated rabbit anti-rat IGg (1:1000, Vector Laboratories. Burlingame, CA).

Animals used for TRH and SP immunostaining were anesthetized with chloral hydrate, then perfused for 5 min with 4% acrolein (Aldrich Chemicals Gold label) in 100 mM phosphate, pH 7.2 ([ref. 23]). Forty-micron Vibratome sections were permeabilized with ethanol (50%, 70%, 50% in PBS) and incubated with primary antisera (TRH 1:1500; SP 1:1000) for 48 h at 4 °C. The TRH was visualized using a single bridge peroxidase–antiperoxidase technique[36] and substance P was visualized by the ABC method

The specificity of the antisera was determined by liquid-phase absorption with appropriate ligand for 24 h prior to application onto tissue sections. Serotonin immunoreactivity was completely blocked by preincubation with 5 μM 6-OH-tetrahydro-β-carboline, a reaction product of serotonin and formaldehyde. Similarly, the binding of the TRH antiserum and SP monoclonal antibody was inhibited by 5 μM TRH or SP, respectively.

Results

Biochemistry

In previous investigations we have demonstrated the permanent depletion of serotonin (>90%) throughout the adult brain following neonatal 5,7-DHT treatment[35]. In this study we have measured the depletion of substance P and TRH in order to determine biochemically the extent of peptide-serotonin co–localization throughout the brain. In adult rats (45 days old), treated neonatally with 5,7-DHT, the TRH content was reduced in the spinal cord (50%) and medulla (20%) (Table I). However, TRH depletion was never as great as serotonin depletion (>90%), nor was any change in TRH content measured in the hypothalamus or midbrain. Thus far the identity of extrahypothalamic TRH has been

somewhat controversial, hence we proceeded to ascertain that, indeed, the TRH immunoactivity in these tissues co-chromatographs with synthetic TRH using reverse-phase HPLC separation[31] (data not shown)

In contrast, substance P content of the dorsal or ventral spinal cord, medulla, midbrain and hypothalamus was not altered by the neonatal 5,7-DHT treatment (Table II). These findings are somewhat surprising, in view of reports[12,24,30] indicating that adult 5,7-DHT treatment results in a substantial loss of substance P in the ventral spinal cord To examine this issue further, we also have replicated the effect of adult 5,7-DHT treatment (day 32) on spinal cord substance P Two weeks after treatment, the ventral spinal cord substance P had declined by 50%, while dorsal spinal cord substance P remained unchanged.

To determine if the substance P in afferent nerve terminals was influencing our results, we treated rats neonatally with capsaicin[19,37] Capsaicin treatment alone led to an 85% decrease of substance P content in the spinal cord. When capsaicin was given in conjunction with 5,7-DHT, no additional loss of substance P was measured in the medulla or spinal cord. Capsaicin alone or in conjunction with 5,7-DHT did not lead to any additional changes in TRH content of any brain region.

Immunocytochemistry

Fixation constituted a crucial factor for effective visualization of TRH, substance P or serotonin. To demonstrate TRH-IR with our antiserum, the tissue must be fixed with acrolein[23], while serotonin could be detected only in tissue fixed with formaldehyde. The serotonin antisera apparently binds to 6-OH-tetrahydro-β-carbohne, a reaction product of formaldehyde and serotonin. Unlike Hokfelt et al.[13,14], we have not been able to demonstrate any TRH-IR in tissues fixed with paraformaldehyde. Fortunately, both acrolein and paraformaldehyde were suitable fixatives for immunocytochemical localization of substance P.

The effect of 5,7-DHT on serotonin lmmunostaining in neurons of the rat ventral medulla is shown in Fig. 1. Following the neonatal treatment, the number of serotonergic neurons and terminals is dramatically reduced throughout the entire brain[35].

Although TRH was only demonstrated in acrolein-fixed tissue, the immunocytochemical pattern of neuronal populations was virtually identical to that reported by Hokfelt et al.[13,14] and Lechan and Jackson[23]. Numerous TRH-IR neurons and fibers were seen throughout the pons-medulla. There is a correlation between substance P cell bodies (Fig. 2A) and the location of TRH-positive cells in the ventral hindbrain (Fig. 2C). In addition, we have observed many TRH-positive cells in the hypothalamus. No TRH-immunoreactive neurons were observed in any other region, including the midbrain and the dorsal and medial raphe nucleus. Following neonatal 5,7-DHT treatment, no intact TRH-IR neurons in the ventral pons-medulla were observed. However, many TRH-IR nerve terminals remained present, particularly in the solitary tract (Fig. 2D and E), and this is consistent with the observation of relatively modest reductions in TRH content following 5,7-DHT treatment. The density of TRH fibers was significantly decreased in the ventral horn of the spinal cord as others have previously demonstrated[12]. Hypothalamic TRH-IR did not appear to be altered following 5,7-DHT treatment.

Substance P – IR was demonstrated in tissue fixed with either acrolein or paraformaldehyde and its distribution appeared identical in tissue prepared with either fixatives. A much larger number of substance P-IR neurons, in comparison to TRH neurons, were seen throughout the brain. In the ventral medulla (Fig. 2A), substance P seems to be located in the same neurons as TRH and serotonin[20,32]. However, we observed only a few substance P neurons

in either the dorsal or medial raphe nucleus. Following neonatal 5,7-DHT treatment, substance P immunoreactivity in neurons of the ventral pons-medulla was completely eliminated (Fig. 2B). Substance P – IR cell groups in other brain regions, such as interpeduncular nucleus, habenula and dorsal pons-medulla, were not affected by the neurotoxin treatment. A group of substance P – IR neurons located slightly lateral to the dorsal raphe nucleus was not eliminated. Following this early treatment, we did not notice any region with significant nerve terminal loss, although given the high density of substance P terminals throughout the brain, it would be difficult to observe any small change.

Discussion

In a previous paper we have characterized the extensive destruction of brain serotonergic neurons, following early neonatal 5,7-DHT treatment[35]. Such treatment results in the cell death of more than 85% of serotonergic neurons, yet catecholamine neurons remain undamaged. Other investigators have demonstrated that treatment of adult rats or the subcutaneous treatment of neonatal rats results in pruning of distal terminals without loss of proximal fibers and serotonergic cell bodies[2], reviewed in [ref. 4]. Since these cell bodies remain intact within the pons-medulla and midbrain, it has not been possible to interpret unambiguously thus far the effect of serotonin depletion on putative peptide content or its co-localization in these brain regions. On the other hand, as serotonergic neurons do degenerate following neonatal 5,7-DHT treatment, it seems unlikely that the surviving peptide neurons contain serotonin. Hence, our 5,7-DHT treatment paradigm may provide biochemical and immunocytochemical data for determination of the relative contribution of co-localized peptide content to the total peptide concentration.

TRH-serotonin co-localization

The present investigation has demonstrated that neonatal 5,7-DHT treatment results in concurrent destruction of certain neuronal populations which contain serotonin, TRH and substance P. Importantly, this study has provided evidence indicating that the amount of either peptide which is co-localized with serotonin constitutes a relatively small portion, compared to the amount of peptide that is not co-localized. The ventral spinal cord appears to be the only brain region where a significant degree of co-localization exists. TRH content declines by 50%, following 5,7-DHT, indicating that at least half the amount of this peptide must be co-localized with serotonin. However, the degree of co-localization is only 20% in the pons-medulla, and in rostral brain areas, TRH does not appear to be significantly co-localized with serotonin. These findings are consistent with the loss of TRH-IR in the ventral spinal cord, although some TRH fibers remain intact, particularly dorsal to the spinal canal. The modest effect of the neurotoxin on medullary TRH content is consistent with the presence of many TRH-IR terminals throughout the hindbrain. In general, as detected by our technique, there are few remaining TRH-IR cells in any CNS region, with the exception of the hypothalamus, raising the possibility that the TRH-IR terminals may either be of hypothalamic origin or alternatively they may enter the brain from peripheral sensory ganglia. In either case, it seems clear from these data that a major portion of TRH is not co-localized with serotonin, and TRH which is co-localized must arise entirely from serotonergic neurons of the pons-medulla and not midbrain.

Substance P–serotonin co-localization

The co-localization of serotonin and substance P appears to be more complex, since the serotonin neurons of the ventral pons-medulla are destroyed, yet substance P content is not decreased following the neurotoxin. Presumably the co-localized substance P represents such a small portion of the total substance P content that the effect of 5,7-DHT could not be reliably determined. Most substance P-containing neurons are in brain regions which do not

contain serotonergic neurons[15,26], and they appear to be unaffected by the neonatal 5,7-DHT treatment. As in the case of TRH co-localization, cells of the ventral pons-medulla are the only serotonergic neurons which also seem to contain substance P. We have not observed any evidence for substance P in serotonergic cells of the dorsal or medial raphe nucleus or in terminals projecting rostrally. Previously, Chan-Palay[9] observed that treatment of adult rats with 5,6-dihydroxytryptamine led to the loss of serotonin–substance P cells in the ventral medulla. Unlike our study, the potential for damage to catecholamine neurons was present, since animals were not pretreated with the catecholamine uptake inhibitor, desipramine

Further, our data have provided additional evidence for taxonomic separation of serotonergic neurons in the midbrain and pons-medulla on the basis of peptide content. Midbrain serotonergic neurons (including the DRN and MRN) do not appear to contain any detectable amount of substance P or TRH. Conversely, the serotonin neurotoxin appears to have no effect on any substance P or TRH neurons anterior to the pons. In contrast, all neurons in the ventral pons-medulla which evidently co-localize substance P, TRH and serotonin, are susceptible to 5,7-DHT, indicating that these neurons probably share some common phenotypic characteristics. Olson and Seiger[28] and Wallace et al.[36] reported the differential origin of medullary and midbrain serotonergic neurons from two separate precursor zones The B_1–B_3 cell groups arise from the embryonic medulla on embryonic day 14, whereas midbrain cells arise nearly 2 days later from a region rostral to the pontine flexure. Differences of developmental timing and origin are likely to lead to the selective distribution of peptides in neurons which contain serotonin.

The effect of 5,7-DHT treatment in adult rats on substance P and TRH immunocytochemistry and content was recently described by Gilbert et al [12] These investigators reported that there was a concomitant decline in serotonin and both peptides in the ventral spinal cord. However, we have not observed a parallel between serotonin depletion and substance P or TRH content in the spinal cord or any other brain tissue, following neonatal 5,7-DHT treatment. Tessler et al.[33] have demonstrated substance P recovery in the cat, following unilateral spinal deafferentation, thus raising the possibility of reinnervation of depleted regions.

Alternatively, one may be measuring different peptides with the different antisera used in these studies. The monoclonal substance P antibody has been well characterized by Cuello et al.[10] and its appears to be specific for the carboxyl terminus of the peptides. However, substance P assays using this antibody give peptide concentration values which are less than those determined with assays based on polyclonal substance P antisera[10]

Neonatal 5,7-DHT treatment has provided a method for eliminating virtually all medullary serotonergic neurons, including those that co-store substance P and TRH. Although we have found modest changes of peptide distribution and content in the medulla, we have also observed apparent long-term supersensitivity to exogenously applied peptides Respiratory responses to intracisternal TRH in halo-thane-anesthetized rats are significantly enhanced, following neonatal 5,7-DHT treatment[27]. Moreover, in depleted animals, the [^{125}I]TRH binding is greater in the spinal cord and medulla, but not in the hypothalamus[29].

Taken together, these different lines of evidence indicate that the neonatal 5,7-DHT paradigm can serve as a useful model system for the study of co-localization of serotonin and peptide neurotransmitters and for an elucidation of the functional significance of this phenomenon

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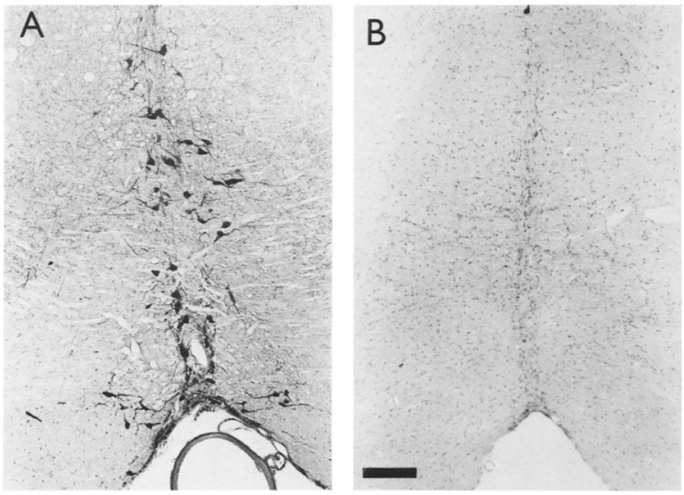

Fig 1.
Serotonin immunocytochemistry in the rat ventral medulla A: control B: neonatal 5,7-DHT + DMI Bar = 100 μm.

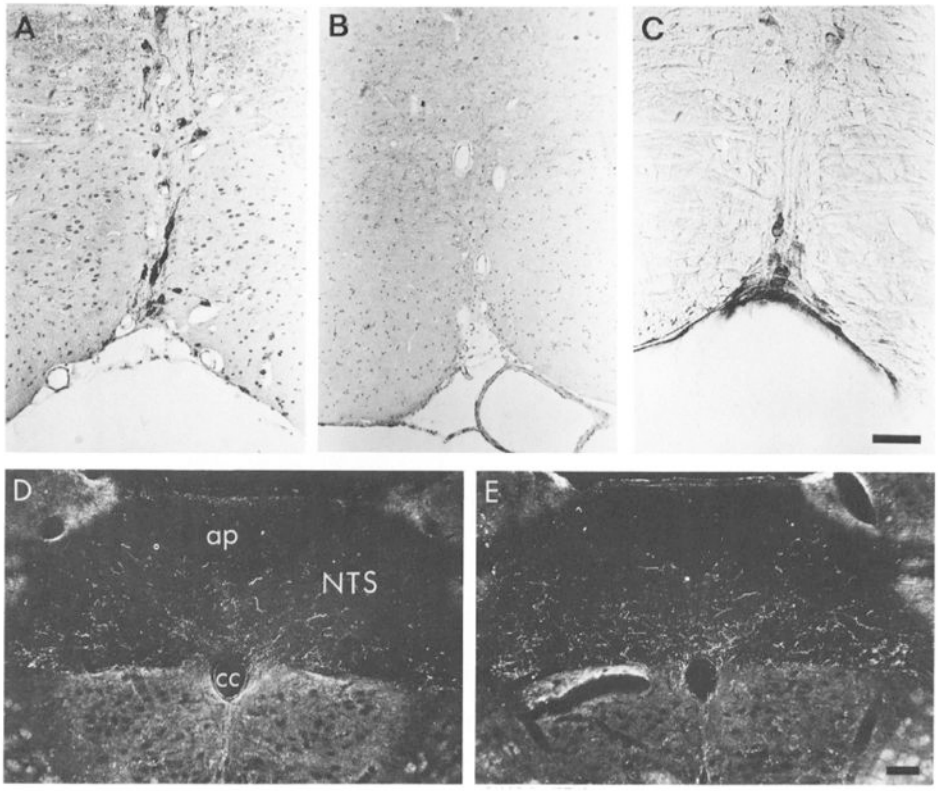

Fig 2.

A substance P immunoreactivity in the medulla of control rat. B: substance P immunoreactivity in medulla of 5,7-DHT-treated rat. C: TRH-immunoreactivc cells in the medulla D TRH immunoreactivity in control rat. E TRH immunoreactivity in 5,7-DHT-treated rat. Bar = 100 μm. ap, area postrema; cc. central canal: NTS. nucleus tractus solitaris A and B were counterstained with toluidine blue, and D and E were photographed using dark-field illumination

TABLE I

TRH content of several brain regions following neonatal 5,7-DHT + DMI and/or capsaicin treatment

Rats were treated as described in Materials and Methods n = 12 Data expressed as X̄ ± SEM

Treatment	Spinal cord	Pons-medulla (ng/mg protein)	Midbrain
Saline	0 98 ± 0 15	0.41 ± 0 08	0 24 ± 0 04
5,7-DHT + DMI	0 43 ± 0 11*	0 25 ± 0 05*	0 20 ± 0 05
Capsaicin	0 85 ± 0 12	0 47 ± 0 05	0 29 ± 0 03
5,7-DHT + DMI + capsaicin	0 45 ± 0 08*	0 27 ± 0 04*	0 31 ± 0 06

* $P < 0\ 05$ when compared to saline

TABLE II

Substance P content of several brain regions following neonatal 5,7-DHT + DMI and/or capsaicin treatment

Rats were treated as described in Materials and Methods n = 12 Data expressed as$\bar{X}$ + SEM

Treatment	Spinal cord	Pons-medulla (ng/mg protein)	Midbrain
Saline	7 70 ± 1 05	0 86 ± 0 11	0 71 ± 0 09
5,7-DHT + DMI	8 40 ± 0 81	0 76 ± 0 09	0 79 ± 0 15
Capsaicin	1 15 ± 0 25*	0 60 ± 0 04*	0 84 ± 0 06
5,7-DHT + DMI + capsaicin	1 27 ± 0 17*	0 64 ± 0 05*	0 73 ± 0 08

*$P < 0\ 05$ when compared to control