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Time Course of Changes in Nociception After 5,6-Dihydroxytryptamine Lesions of Descending 5-HT Pathways[1]

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BERGE, O.-G., O. B. FASMER, T. FLATMARK AND K. HOLE. *Time course of changes in nociception after 5,6-dihydroxytryptamine lesions of descending 5-HT pathways.* PHARMACOL BIOCHEM BEHAV **18**(4) 637–643, 1983.—Intrathecal injection of 5,6-dihydroxytryptamine (5,6-DHT) in rats produced selective lesions of the descending 5-HT pathways. Spinal 5-HT levels gradually fell to less than 10% of controls within 10 days of 5,6-DHT administration with no recovery evident within 4 weeks. The uptake of ^{14}C-5-HT into crude spinal synaptosomes was similarly reduced. The uptake of ^{3}H-NA into spinal synaptosomes was unaffected, as was the uptake of ^{14}C-5-HT and ^{3}H-NA into cortical synaptosomes. Following 5,6-DHT, tail-flick latencies were reduced by 20–30% during the first post-injection week, but returned to control levels during the second week. Intrathecal or systemic administration of the 5-HT receptor antagonist metergoline significantly reduced latencies of normal rats and of 5,6-DHT treated rats tested after the second week when the response was normalized. Metergoline did not, however, further reduce the latencies of lesioned rats during the first post-injection week. It is concluded that functional adaptation involving 5-HT neurotransmission compensated for the selective lesion of descending 5-HT pathways induced by 5,6-DHT.

5-Hydroxytryptamine 5,6-Dihydroxytryptamine Metergoline Selective lesioning Nociception Functional recovery Tail-flick response Rat

IN rats, nociception measured by the tail-flick response to noxious radiant heat is subject to tonic inhibition probably involving the raphe-spinal 5-hydroxytrypaminergic (5-HT) pathways [6, 7, 27, 37]. We previously suggested that activity of descending 5-HT neurons is depressed by presynaptic stimulation, since intracerebroventricular injections of low doses of the 5-HT receptor agonist 5-methoxy-N, N-dimethyltryptamine induced hypersensitivity in the tail-flick test [9]. Similar mechanisms have been described for the ascending 5-HT systems [11,35]. Distinguishing between interference with descending activity and direct post-synaptic actions therefore becomes a problem in interpreting drug effects in relation to the raphe-spinal 5-HT system. This problem may be circumvented by using chronically spinalized animals [6,8]. In this model, spinal nociceptive reflexes may be studied in the complete absence of descending nervous control. A technique for selective lesioning of the descending 5-HT pathways would, however, be useful for investigating the role of spinal 5-HT receptors in modulating reflexes as well as complex behaviors elicited by noxious stimulation.

Electrolytic lesions in the medulla oblongata [26] and intracerebroventricular injections of neurotoxins [36] have previously been used to disrupt descending 5-HT pathways. These methods also damage non-5-HT structures or ascending 5-HT pathways, making the results ambiguous in terms of neurotransmitter systems involved. Recently, injection of the neurotoxin 5,6-dihydroxytryptamine (5,6-DHT) into the lumbar subarachnoidal space has been introduced as a selective method for lesioning of the raphe-spinal serotonergic system [28,37]. The treatment reduced response latencies to noxious heat, in agreement with the hypothesis that descending 5-HT pathways mediate tonic depression of nociception. The increased reactivity was, however, of short duration, lasting less than 14 days.

In the present study, we investigate the effect of similar injections on the tail-flick response of rats. The extent of damage to descending and ascending 5-HT neurons as well as to noradrenergic pathways is evaluated by studying the *in vitro* uptake of labeled neurotransmitters into synaptosomal fractions prepared from homogenates of lumbar spinal cord and cortical tissue. The endogenous level of spinal 5-HT is also monitored. Particular attention is paid to the time course of the behavioral and biochemical changes induced by the lesions. Furthermore, the 5-HT receptor antagonist metergoline is administered intrathecally during recovery in order to investigate the degree of 5-HT involvement in the sensitivity changes observed during this period.

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METHOD

Animals

A total of 140 male Wistar rats (Møllegård, Denmark, weight 260–290 g) were individually housed in conventional cages with free access to food and water.

Materials

Serotonin (5-hydroxytryptamine creatinine sulfate monohydrate) was obtained from the Sigma Chemical Company, 5,6-DHT (5,6-dihydroxytryptamine creatinine sulfate) from Regis Chemicals, ^{14}C-5-HT (^{14}C-5-hydroxytryptamine creatinine sulfate) and ^{3}H-NA (dl-^{3}H-noradrenaline hydrochloride) from The Radiochemical Centre, Amersham. Metergoline was donated by Farmitalia. All other chemicals were obtained from various commercial sources and were of analytical grade.

Surgery

Surgical anesthesia was induced by a combination of pentobarbital (40 mg/kg) and chloral hydrate (130 mg/kg) given IP. Intrathecal catheterization was carried out according to a previously described method [20] with minor modifications. Briefly, 9.5 cm of polyethylene tubing (PE10), previously stretched just short of breaking, was inserted through a slit in the atlanto-occipital membrane into the subarachnoid space. The free, unstretched end of the catheter was then passed through two holes in the skull, drilled in the midline just rostral and caudal to the occipital crest. The catheter was fixated in rostro-caudal direction by a bead of cranioplastic cement located in the angle between the atlanto-occipital membrane and the occipital bone. Due to the stretching of the main portion of the tubing, the total dead-space of the catheter was reduced to less than 4.5 μl.

At least 7 days were allowed for recovery before injection of 5,6-DHT. Distilled water containing 0.2 mg/ml ascorbic acid was used as a vehicle, and 10 μl of solution containing 20 μg 5,6-DHT (calculated as the base) was injected. The catheter was finally flushed with 4.5 μl artificial cerebrospinal fluid (CSF [22]).

A total of 74 rats received 5,6-DHT injections. Thirty-six of these were used for biochemical studies only. Control rats received the same treatment except that 5,6-DHT was omitted from the solution.

Biochemical Analyses

All rats were killed by decapitation during the third or fourth hour of the light period. The brains and lumbar spinal cords were rapidly removed and cooled.

Crude synaptosomes were prepared from approximately 150 mg of tissue from the lumbar spinal cord or from the frontal cortex. The tissue was homogenized in 10 vol of 0.25 M sucrose. The homogenate was centrifuged (1000 ×g, 0°C 10 min), and 200 μl (spinal cord) or 100 μl (cortex) of synaptosome-containing supernatant was added to modified Krebs-Ringer bicarbonate buffer [18] to make a final volume of 2.0 ml. After preincubation at 37°C for 5 min, ^{14}C-5-HT (58.8 mCi/mmol) and ^{3}H-NA (9.0 Ci/mmol) was added to give a final concentration of 1×10^{-8} M of the NA-isotope and 1×10^{-7} M for the 5-HT-isotope. Incubation was continued for another 5 min and terminated by rapid cooling followed by centrifugation (40000 ×g, 0°C, 15 min). The pellets were washed in ice-cold saline and dissolved in Protosol (New England Nuclear). Scintillation fluid (5.5 g Permablend III, Packard, dissolved to 1 l in toluene) was added and the samples analysed in a Packard Tri-Carb 460 CD liquid scintillation spectrometer. Each determination was carried out in triplicate. Uptake determined in presence of cocaine (3×10^{-4} M) was subtracted from the total uptake. ^{14}C-5-HT and ^{3}H-NA activity was separated as described by others [29].

Endogenous levels of 5-HT in the lumbar spinal cord was determined by the following procedure. Approximately 150 mg of spinal tissue was homogenized in an all-glass microhomogenizer. Perchloric acid (0.4 M) containing 0.05% (w/v) disodium EDTA was used as homogenization medium (0.25 g tissue/ml medium). The homogenate was centrifuged at 40000 ×g, 0°C, for 30 min. Fifty μl of the supernatant was immediately analysed by high-performance liquid chromatography with fluorescence detection as previously described [14].

Testing of Nociception

The tail-flick response was tested as described earlier [9], using an IITC Inc. Mod. 33 Analgesiameter, in which radiant heat was focused on a spot 1–2 cm from the tip of the tail. Beam intensity was adjusted to give a reaction time of 3–4 sec in control animals. Testing took place in the middle of the light period of a 12/12 hr light/dark cycle.

All animals were handled and trained in the test-situation before the start of the experiments and between experiments. During each session, the rats were tested at 20 min intervals. When the acute effect of 5,6-DHT was investigated, the drug was injected 10 min after the third trial and testing continued at 20 min intervals for a total of 8 trials, and then with longer intervals until 6 hrs after injection. Similarly, 20 min trial intervals were used when testing the effects of the 5-HT receptor antagonist metergoline [15,30]. Metergoline was administered as 4 μg in 5 μl CSF containing 10% 0.1 M methansulfonic acid for intrathecal injections or as 0.25 mg/kg in 1 ml/kg saline containing 5 mg/ml ascorbic acid for intraperitoneal injections. Intraperitoneal injections were carried out immediately after the third trial and intrathecal injections started 10 min after the third trial. Intrathecal injections were performed by means of a microsyringe driven by a perfusion pump at a rate of 6 μl/min. The implanted catheter was connected to the microsyringe by polyethylene tubing (PE50), allowing the animals to be unrestrained in the home-cage during injection.

Following lesioning, basal levels of tail-flick latencies were recorded daily as the mean score of trial 2 and 3 in a 3-trial session.

Statistics

Analysis of variance (ANOVA) was applied to the data from all experiments.

RESULTS

Catheterization had no observable effect on motor performance or tail-flick response. Histological examination of spinal cords from a series of rats previously catheterized and lesioned according to the methods described here revealed only minor changes, similar to those reported by others [20].

Biochemical Evaluation of the Lesions

Injections of 5,6-DHT reduced the uptake of ^{14}C-5-HT in spinal cord synaptosomal preparations to approximately 80%

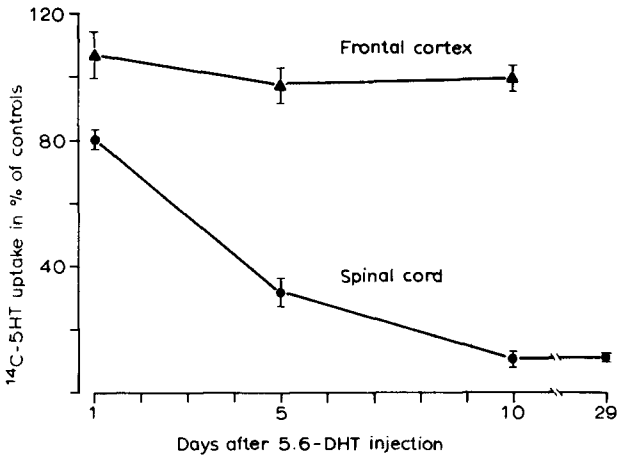

FIG. 1. Uptake of ^{14}C-5-HT into cortical and spinal crude synaptosomes from 5,6-DHT treated rats. Mean±S.E.M. of determinations made in triplicate (n=4–6 for each point for days 1, 5 and 10, n=19 for day 29).

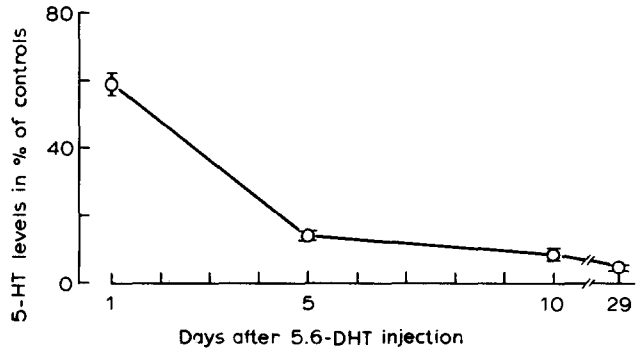

FIG. 2. Spinal levels of 5-HT in 5,6-DHT treated rats. Mean± S.E.M. (n=7 for each point). Control level determined in vehicle-injected rats was 0.69±0.02 μg/g.

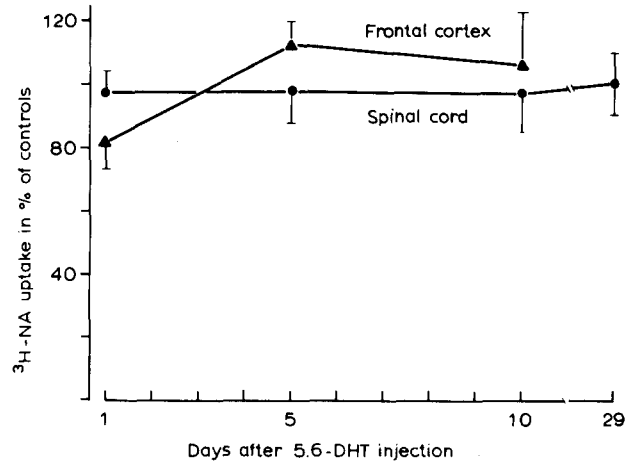

FIG. 3. Uptake of ^{3}H-NA into cortical and spinal crude synaptosomes from 5,6-DHT treated rats. Mean±S.E.M. for determinations made in triplicate (n=4–6 for each point for days 1, 5 and 10, n=19 for day 29).

of controls within 24 hrs, and to 32% and 10% within 5 and 10 days, respectively (Fig. 1). Uptake studies carried out in the animals used for tail-flick testing revealed no indication of regeneration up to the 4th week after injection (uptake determined at day 29: 11±1.5% of controls, mean±S.E.M., n=19).

The endogenous level of 5-HT determined in the spinal cord of control animals was 0.69±0.02 μg/g ww (mean± S.E.M., n=13). After 5,6-DHT administration, the levels were initially reduced at a somewhat more rapid rate than the uptake (Fig. 2), but the reductions observed on day 10 and 29 were nearly identical in the two assays.

No statistically significant changes in uptake of ^{14}C-5-HT into cortical synaptosomal preparations (Fig. 1) or ^{3}H-NA into spinal or cortical synaptosomal preparations (Fig. 3) were found, although minor changes in ^{3}H-NA uptake into the cortical preparations were present.

Thus, the biochemical evaluation demonstrated that significant, selective lesions of the descending 5-HT pathways were obtained by the 5,6-DHT treatment. There was no indication of regeneration within the time-period investigated.

The Effect of 5,6-DHT Administration on Nociception

Thirty-five previously catheterized rats were randomly assigned to two groups. There was no difference in tail-flick latencies between the groups before intrathecal injection of 5,6-DHT (Vehicle group: 3.6±0.1 sec, n=18; 5,6-DHT group: 3.7±0.1 sec, n=17; mean±S.E.M. for 3 pooled preinjection trials). Administration of 5,6-DHT was immediately followed by transient limping and occasional vocalization which faded before the first post-injection tail-flick test. No reaction was observed to the injection of vehicle.

In comparison with the vehicle group, the 5,6-DHT injected rats showed a significant elevation of tail-flick latencies to 153±13% 10 min after injection, $t(33)=3.75$, $p<0.001$. The elevation was of short duration and gave way to a gradual reduction in latencies. The mean latencies for the trials taking place between 1 and 6 hrs after injection were between 80 and 95% of controls. ANOVA for repeated measures applied to the scores obtained from the 6 trials during this period revealed significant difference between the groups, $F(1,33)=10.18$, $p<0.005$ but not between trials, $F(5,165)=1.97$ or for interaction, $F(5,165)=1.45$.

The tail-flick latencies remained short for several days following 5,6-DHT administration (Fig. 4). The effect was most pronounced 24 hrs after injection (71% of control latencies) and the difference between 5,6-DHT treated animals and controls gradually diminished during the second post-injection week. No significant difference was observed on day 15 and day 20, whereas a slight, nonsignificant elevation was recorded for the 5,6-DHT group on tests taking place between 3 and 5 weeks after injection (data not shown). ANOVA applied to the basal level scores shown in Fig. 4 and similar data obtained during the third and fourth week after injection demonstrated highly significant effects ($p<0.001$) between groups, $F(1,33)=28.33$, trials, $F(14,462)=5.11$ and for interaction, $F(14,462)=6.11$.

The Effect of Metergoline on Nociception

Separate groups of animals were used to study the effects of 5-HT receptor blockade by metergoline. Basal tail-flick

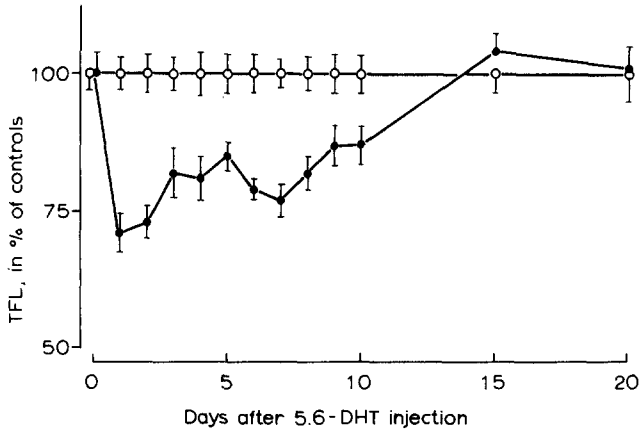

FIG. 4. Effect on nociception of 20 μg 5,6-DHT (n=17, closed circles) or vehicle (n=18, open circles) given intrathecally. Data presented as mean±S.E.M.

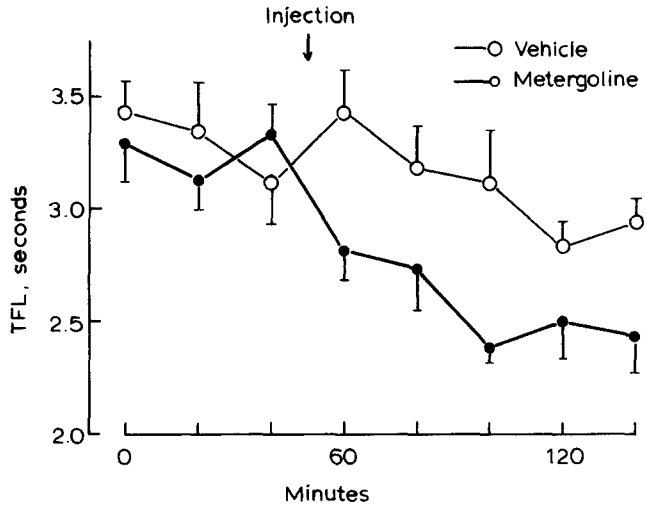

FIG. 5. Effect on nociception of intrathecal injection of vehicle and of the 5-HT receptor blocker metergoline in non-lesioned rats (n=9–10 for each group). Data given as mean±S.E.M.

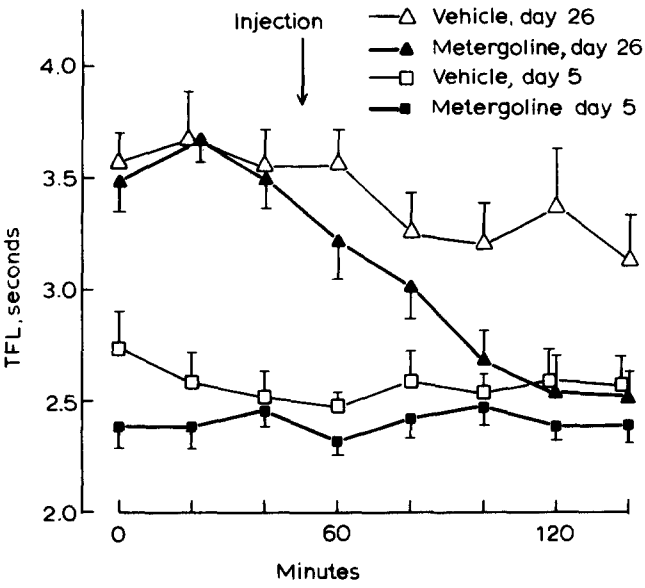

FIG. 6. Effect on nociception of intrathecal injection of vehicle and of the 5-HT receptor blocker metergoline in 5,6-DHT lesioned rats (n=8–10 for each group). Data given as mean±S.E.M.

latencies were recorded less frequently from these rats, but the values obtained from 5,6-DHT lesioned rats followed the same time course as described above.

In animals not previously given 5,6-DHT, intrathecal injection of 4 μg metergoline significantly reduced the tail-flick latencies for more than 90 minutes (Fig. 5). ANOVA demonstrated significant effects between metergoline and vehicle-injected groups, $F(1,17)=6.22$, $p<0.025$, trials, $F(7,119)=7.54$, $p<0.001$ and of interaction, $F(7,119)=2.29$, $p<0.05$. There was no difference between the group scores prior to the injections.

When given 5 days after 5,6-DHT, metergoline did not alter the already reduced tail-flick latencies of the lesioned rats, Fig. 6; groups: $F(1,17)=1.89$, $p>0.10$; trials: $F(7,119)<1.00$; interaction: $F:7,119)<1.00$; ANOVA for metergoline vs. vehicle treated lesioned rats.

The effect of metergoline intrathecally was also tested 26 days after 5,6-DHT administration, when the tail-flick latency had returned to normal (Fig. 6). At this time, the 5-HT receptor antagonist induced a reduction in tail-flick latency similar to that observed in controls. ANOVA demonstrated significant main effects between metergoline and vehicle injected groups, $F(1,14)=5.49$, $p<0.05$, trials, $F(7,98)=10.06$, $p<0.001$ and for interaction, $F(7,98)=2.25$, $p<0.05$. There was no significant difference between the groups prior to injection of metergoline or vehicle.

Similarly, systemic (IP) administration of metergoline on day 14 reduced the tail-flick latencies to approximately 75% of preinjection values in lesioned animals as well as in controls (Table 1). ANOVA (2×2 factorial design with pre and post injection scores as repeated measures) confirmed that there was no significant difference between the tail-flick latencies of 5,6-DHT injected animals and controls at this stage, lesion effect: $F(1,29)<1.00$. The difference in latencies between metergoline and vehicle-injected rats was, however, significant, metergoline effect: $F(1,29)=10.06$, $p<0.005$. There was no tendency towards interaction between these factors, $F(1,29)<1.00$. The reduction in latencies 50 min after injection was highly significant, trial effect: $F(1,29)=20.35$, $p<0.001$, with reliable interaction present between trial and metergoline effects, $F(1,29)=14.94$, $p<0.001$, but not between trial and lesion or trial, metergoline and lesion effects, $F(1,29)<1.00$ for each interaction.

Thus, in control animals, and in 5,6-DHT lesioned rats in which the tail-flick response had returned to normal, blockade of 5-HT receptors by systemic or intrathecal metergoline administration consistently reduced the tail-flick latencies. There was on the other hand no further reduction in latencies of the lesioned rats during the period of hyperreactivity.

DISCUSSION

The present data show that extensive lesioning of descending 5-HT pathways may be obtained by intrathecal

TABLE 1

THE EFFECT OF SYSTEMIC INJECTION OF METERGOLINE ON THE TAIL-FLICK RESPONSE OF 5,6-DHT TREATED ANIMALS AND CONTROLS

Group	Treatment	Response Latencies	
		Preinjection	Postinjection*
Control (8)	Vehicle	3.1 ± 0.2	3.1 ± 0.2
Lesion† (7)	Vehicle	3.3 ± 0.1	3.3 ± 0.2
Control (10)	Metergoline	3.2 ± 0.1	2.4 ± 0.2
Lesion (8)	Metergoline	3.3 ± 0.2	2.5 ± 0.1

Tail-flick latencies are given in seconds as means ± S.E.M. Number of animals in parentheses.

*Tested 50 min after IP injection of vehicle or 0.25 mg/kg metergoline.

†Lesions produced by injection of 20 μg 5,6-DHT intrathecally 14 days prior to testing.

5,6-DHT injections. The treatment reduced endogenous levels of 5-HT in the lumbar spinal cord and the *in vitro* uptake of ^{14}C-5-HT into spinal synaptosomal preparations by about 90%. The lesions did not affect NA systems or ascending 5-HT systems as monitored by the synaptosomal uptake of neurotransmitters, although catecholaminergic structures are particularly vulnerable to the general cytotoxic effects of 5,6-DHT [3, 4, 10]. These results demonstrate that the lesions were rather selective for the descending 5-HT pathways. It should be remembered, however, that this system contains most of the spinal substance P of non-primary afferent origin [12, 17, 31]. TRH may also coexist with 5-HT in spinal terminals [19]. It is therefore likely that the lesions decreased the content of these putative transmitters as well.

The biochemical data do not provide any evidence for regeneration of the lesioned 5-HT terminals within 4 weeks of 5,6-DHT injection. On the other hand, the increased responsiveness to noxious heat gradually disappeared within 2 weeks, confirming the results of others [28]. This finding indicates that a functional adaptation took place, compensating for the reduction in spinal 5-HT innervation. Apparently, the compensation processes involve spinal 5-HT transmission. The 5-HT receptor antagonist metergoline had no effect on the tail-flick response when administered intrathecally 5 days after lesioning, when the reactivity was still increased. In lesioned rats after recovery, intrathecal as well as systemic administration of metergoline significantly reduced the tail-flick latency to the same extent as in non-lesioned rats.

Metergoline is a very potent 5-HT antagonist in the tail-flick test [6], but it is unlikely that any significant quantity of the intrathecally injected drug might have reached supraspinal sites. In addition, previous work has indicated that the effect of systemically injected metergoline in the tail-flick test is due to spinal actions of the drug [6]. Thus, it appears that the descending 5-HT system in the rat spinal cord may eventually compensate for the major lesions (90% reduction of spinal 5-HT levels or synaptosomal uptake), in a way that restores normal tonic inhibition of nociceptive reflexes. Similar observations of functional recovery have previously been made with regard to behavioral effects of lesions of the ascending pathways [16]. Biochemical evaluations do not suggest that other techniques for selective lesioning of monoaminergic pathways are appreciably more effective than the one used in the present study. The possibility of functional regeneration should therefore always be considered in behavioral studies using lesions of these pathways.

Evidence derived from receptor binding [16], microiontophoretic [13, 21, 25] and behavioral [32–34] studies have demonstrated supersensitivity to 5-HT after neurotoxic lesions of serotonergic pathways. The functional recovery observed in the present experiments may at least partly be a result of similar supersensitivity developing in postsynaptic spinal receptors. This explanation is supported by the temporal similarity between the return of normal nociception in the present experiment and the increase in sensitivity to 5-HTP-induced EMG activity reported by others after spinal transection [1, 2, 5]. Furthermore, when the descending 5-HT pathways are totally severed by spinal transection, lower doses of 5-HT agonists are needed to depress the tail-flick response [6]. Basal level tail-flick latencies remain short for at least 36 weeks, however, indicating that a minimum of descending innervation is necessary to normalize the response.

On this basis it is evident that great care should be taken when interpreting the effects of lesions in 5-HT systems. The duration of the period allowed for recovery after lesioning may be particularly critical. In the present study, maximal reduction in synaptosomal uptake was reached between 5 and 10 days after 5,6-DHT and remained at approximately 10% of controls for at least 29 days. The depletion of endogenous 5-HT followed rougly the same pattern. The tail-flick response was on the other hand affected immediately. The injection was followed by a transient decrease in sensitivity which may partly be due to release of 5-HT from the affected terminals, combined with a direct receptor agonist action of 5,6-DHT [23]. Within 1 hr of injection, however, the effect was reversed, and latencies below control levels were recorded. The peak reduction was observed 24 hrs after lesioning, when the synaptosomal uptake was still 80% and 5-HT levels 60% of controls. The tail-flick latency returned to control levels by 15 days, when the uptake and levels were reduced by 90% or more.

The reduction in tail-flick latency probably reflects a functional disruption of the descending 5-HT system as accumulated 5,6-DHT interferes with the metabolism and function of the fibers and terminals. The synaptosomal uptake on the other hand reflects the condition of the transmitter reuptake mechanism which is not dependent on the integrity of the entire neuron. Following 5,6-DHT, 5-HT containing terminals degenerate less rapidly than axons [24], with a rate of disappearance that parallels the reduction in uptake reported in the present study. It is therefore reasonable to assume that the long-term effects of 5,6-DHT on synaptosomal uptake reflects the extent of irreversible structural damage to the descending 5-HT system.

In conclusion, intrathecal injection of 5,6-DHT selectively lesioned descending 5-HT pathways. The lesions blocked the inhibitory function of the raphe-spinal system on nociception, but only for a limited time although no structural regeneration was detected by the synaptosomal uptake method. Determination of spinal 5-HT levels and synaptosomal uptake on day 1 and day 5 after lesioning apparently underestimated the degree of functional lesion during this period. Within two weeks, adaptation had taken place so that normal inhibitory function was observed in the tail-flick test.

Thus, when tested in the first post-injection week, 5,6-DHT treated animals apparently lack a functioning descending 5-HT system. The model could therefore prove useful in

behavioral studies. Interpreting the effects of drugs given during this period could, however, be complicated by the possibility of presynaptic interactions with the degenerating structures.

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