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Lesions of the Ascending Serotonergic Pathways and Antinociceptive Effects After Systemic Administration of p-Chloroamphetamine in Mice

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HUNSKAAR, S , O -G BERGE, O J BROCH AND K HOLE *Lesions of the ascending serotonergic pathways and antinociceptive effects after systemic administration of p-chloroamphetamine in mice* PHARMACOL BIOCHEM BEHAV **24**(3) 709–714, 1986 —The present study reports a method for lesioning of the ascending serotonergic system The neurotoxic substance p-chloroamphetamine (PCA) was given IP on 2 consecutive days (40 mg/kg/day) After each injection, the animals were kept at 4°C for 4 hr since a lower dose of PCA (25 mg/kg) induced severe hyperthermia The mortality rate was 12%, considerably lower than previously reported in similar studies Evaluated 9 days after the last injection of PCA, the uptake of ^{14}C-5-HT into cortical and hippocampal crude synaptosomal preparations was reduced by 50 and 60%, respectively, while the uptake into spinal synaptosomes was unaffected The uptake of ^{3}H-NA was not significantly altered in any of the structures Measurements of PCA performed 30 min to 4 hr after IP injections of 5 to 40 mg/kg demonstrated higher concentrations in the cortex than in the lumbar spinal cord Administration of PCA (5 mg/kg) had an acute antinociceptive effect in the hot-plate and formalin tests, but not in the tail-flick test Prior treatment with neurotoxic doses of PCA prevented the antinociception but had in itself no effect on the responsiveness in any of the tests Thus systemic administration of PCA produces highly selective and functional lesions of the ascending serotonergic pathways in mice

p-Chloroamphetamine Analgesia 5-Hydroxytryptamine Test-dependent effects Tail-flick Hot-plate Formalin test Selective lesions Hyperthermia

THE neurotoxin p-chloroamphetamine (PCA) has been a useful tool for studying the physiological role of serotonin (5-HT) in the CNS of the rat Systemic administration of PCA in the rat has a biphasic effect with an initial release of 5-HT from serotonergic terminals followed by a pronounced and long-lasting decrease in the brain levels of 5-HT associated with lesions of the ascending serotonergic pathways [16, 25, 26] In mice, however, it has been difficult to demonstrate long-term neurotoxic effects of PCA Although a biphasic pattern of 5-HT depletion has been demonstrated in mice as well, the duration of the phases were much shorter in this species and brain 5-HT levels were restored within 3 weeks of administration of relatively high doses of PCA [28] Furthermore, the acute toxicity of PCA limits the amount of drug that can be injected on a single occasion [15, 19, 28]

Continuous release of PCA for 3 days from a subcutaneous mini-pump was shown to decrease the 5-HT levels in mice for at least 4 weeks [29] and it seems that the improved neurotoxic effect was dependent upon the relatively long duration of exposure to the drug It is known that there are different pathways for the metabolism of amphetamine and its derivatives in rats and mice, resulting in species differences in the half-life of PCA [18]

The acute toxicity of PCA has been demonstrated to be a function of several variables, social, pharmacological and environmental modulators have been explored Effects on body temperature may be of particular importance [15] Lethal hyperthermia has previously been reported in rabbits [24], whereas in rats, both hypo- and hyperthermia has been observed [9, 12, 13] Importantly, the effect of PCA on the body temperature of rats appears to vary with the ambient temperature in that a dose of PCA that induced hypothermia at 20°C led to hyperthermia at 25°C [12] Severe hyperthermia as a result of reduced ability for heat dissipation may well explain the high mortality after PCA administration in mice

There is accumulating evidence for a role of 5-HT in nociception Generally, it is suggested that increases in the activity of brain and spinal cord serotonergic pathways are associated with analgesia and that decreases in the activities of these neurons are correlated to hyperalgesia [1,17] Selectively induced lesions of the ascending serotonergic path-

ways by systemic administration of PCA in rats do not, however, alter the nociceptive responses in the hot-plate test [1, 6, 30], the tail-flick test [1,6] or the formalin test [6] In mice, no such data with satisfactory biochemical evaluation of the lesions are available

In rats, acutely administered PCA may induce analgesia in various tests due to the release of 5-HT [21] This analgesia is prevented by prior treatment with PCA in neurotoxic doses [21] It may be assumed that after lesioning there will be no further 5-HT to be released from serotonergic terminals, and thus no antinociceptive effects to be demonstrated A failure of acutely administered PCA to induce analgesia in lesioned animals therefore reflects the functionality of the lesions

In the present study we report a method for lesioning the ascending serotonergic system in mice by means of PCA, taking into account the need for repeated or long exposure and the lethal effect of strong hyperthermia We also investigate the functional effects of the lesions in three different pain tests and test the effects of the lesions on PCA-induced analgesia Measurements of PCA in different brain regions have been performed and the data correlated to the neurotoxic effect of PCA

METHOD

Animals

Male albino mice (NMRI) weighing 30–45 g were used The animals were housed in colony cages with free access to food and water prior to the experiments and were maintained in climate and light controlled rooms (23±0 5°C, 12/12 hour dark/light cycle with lights on at 07 00) for at least 2 weeks prior to the experiments Testing took place during the light phase Prior to the testing the mice were adapted to the testing environment for at least 18 hours

Neurotoxic Lesions

p-Chloroamphetamine hydrochloride (PCA, Sigma Chemicals) was dissolved in 0 9% NaCl and injected intraperitoneally (IP) in a volume of 10 ml/kg In order to produce lesions, PCA was given in doses of 40 mg/kg on two consecutive days The mice were kept at a temperature of 4±1°C for 4 hours after the injections The lesions were evaluated 9 days after the last PCA administration in order to allow complete degeneration of the damaged terminals

Biochemical Analyses

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

Crude synaptosomes were prepared from approximately 150 mg of tissue from the frontal cortex, hippocampus and the lumbar spinal cord The tissue was homogenized in 10 vol of 0 25 M sucrose The homogenate was centrifuged (1000×g, 0°C, 10 min), and 0 075 ml of synaptosome-containing supernatant was added to modified Krebs-Ringer bicarbonate buffer to make a final volume of 0 7 ml After preincubation at 37°C for 3 min, ^{14}C-5-HT and ^{3}H-NA were added to give a final concentration of 10 nM of the NA-isotope and 100 nM of the 5-HT isotope Incubation was continued for another 10 min and terminated by rapid cooling followed by filtration in a Titertec cell harvester in order to collect the synaptosomal specimens The filters were washed with ice-cold saline and put into polyethylene vials with 4 ml scintillation fluid (Insta-Gel, Packard) The samples were analyzed in a Packard Tri-Carb 460°C liquid scintillation spectrometer Each determination was carried out in triplicate Uptake determined in presence of cocaine (0 3 mM) was subtracted from the total uptake

Analysis of PCA in Different Regions of the CNS

The animals were sacrificed and dissected as described above Tissue was obtained from the frontal cortex, brainstem (pons and medulla) and the lumbar spinal cord

PCA was determined with gas chromatography after acetylation and extraction into an organic solvent The acetylation improved the extraction and the chromatographic properties The tissue samples were homogenized in 1 ml 0 4 M $HClO_4$ with 0 1 μg amphetamine added as an internal standard After centrifugation and removal of perchlorate with KOH the neutralized extract was saturated with $NaHCO_3$, and 0 1 ml acetic anhydride was added The tube was shaken gently until no more carbon dioxide was formed The solution was then extracted twice with 5 ml dichloromethane which was combined and evaporated under a stream of nitrogen The residue was dissolved in 50 μl ethyl acetate About 1 μl of the ethyl acetate was injected into a 20 m capillary column (i d 0 25 mm) with methyl silicon phase (OV-1 or SP-255), fitted with an on-column injector and a flame ionisation detector The temperature started at 140°C and increased with 4°C per minute With a gas (Helium) flow of 3 ml/min the retention times were 6 5 min for amphetamine and 10 2 min for PCA on the SP-255 column, or 8 2 and 12 5 min on OV-1 The method is also suitable for determination of amphetamine, using PCA an the internal standard

Body Temperature Measurement

Body temperature was measured in the colon by means of a thermistor probe inserted 3 cm into the rectum of the animals The mice were kept at an ambient temperature of either 4 or 23°C for the duration of the experiment A dose of 25 mg/kg was used for this experiment in order to prevent a significant lethality in the animals kept at room temperature

Nociceptive Tests

Separate groups of animals were used for the various tests The nociceptive testing was performed 6–10 days after the first injection of PCA Each mouse was used on one occasion only Testing was carried out by an observer unaware of the drug treatment

Tail-flick latencies were obtained with 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 about 4 0 sec from the onset of stimulation to the removal of the tail in a sample of untreated animals The hot-plate test was conducted with IITC Inc Mod 35-D Analgesiameter, set to give a temperature of 55±0 2°C Latency to hind-paw lick was recorded In the absence of this response, a cut-off time of 30 sec was observed and assigned as the response latency The mice were adapted to the cold hot-plate for 1–2 min the day before the experiment

A modification of the formalin test originally described by Dubuisson and Dennis [7] was used [14] A 1% solution of formalin in 0 9% NaCl was injected subcutaneously in a volume of 20 μl into the dorsal surface of the right hind-paw of

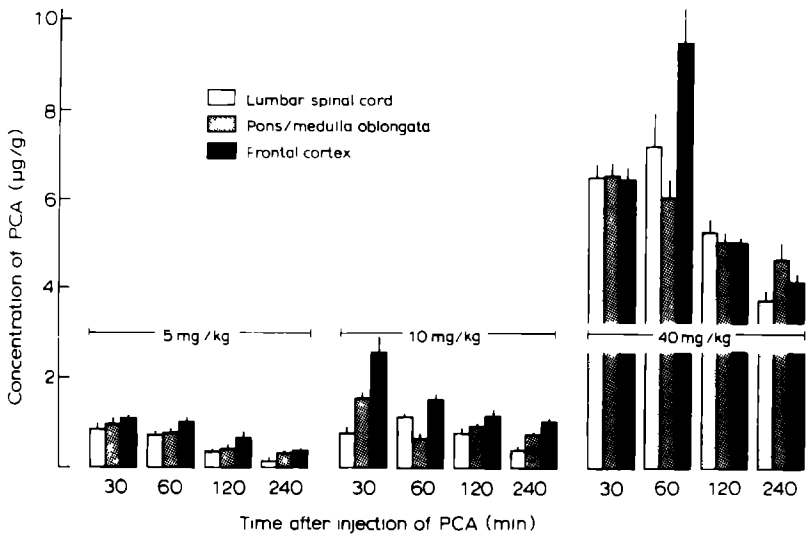

FIG 1 Time course of tissue levels of PCA (µg/g±S E M) in cortex, pons/medulla oblongata and lumbar spinal cord in mice after a single intraperitoneal administration of 5, 10 or 40 mg/kg PCA

the mouse The amount of time the mouse spent licking the injected hind-paw was recorded during the first 2 min after formalin injection The maximal response was thus 120 sec Two hours before testing, the animals were placed individually in standard macrolone cages (30×12×13 cm) which also served as observation chambers There was no water or food available during testing

Statistics

The effect of the PCA treatment was examined by analyses of variance (ANOVA) as detailed in the results Scheffe's test was used subsequent to ANOVA in some experiments When the analyses were restricted to two means, Student's *t*-test for independent measures was performed Unless otherwise stated, significance was accepted at the 5% level

RESULTS

Preliminary experiments suggested that a dose of 20 mg/kg p-chloroamphetamine (PCA) did not cause reductions in the 5-HT uptake in mice and that a dose of 60 mg/kg PCA increased the lethality without improving the lesions (data now shown) The chosen dose of 2×40 mg/kg gave an overall lethality in the present experiments of less than 12% when the mice were housed in cool (4°C) environment In a pilot study, the initial lethality was approximately 50% after 40 mg/kg and the study was discontinued for ethical reasons Thus no lethality experiments per se were performed

Tissue levels of PCA After Single Intraperitoneal Administration

Between 30 min and 4 hr after intraperitoneal injection of PCA, higher concentrations of the compound were found in frontal cortical tissue than in the lumbar cord (Fig 1) For each dose level separate ANOVAs (4 time points by 2 brain regions) were performed The data from the two regions were considered as dependent variables ANOVA showed that the differences between structures were statistically

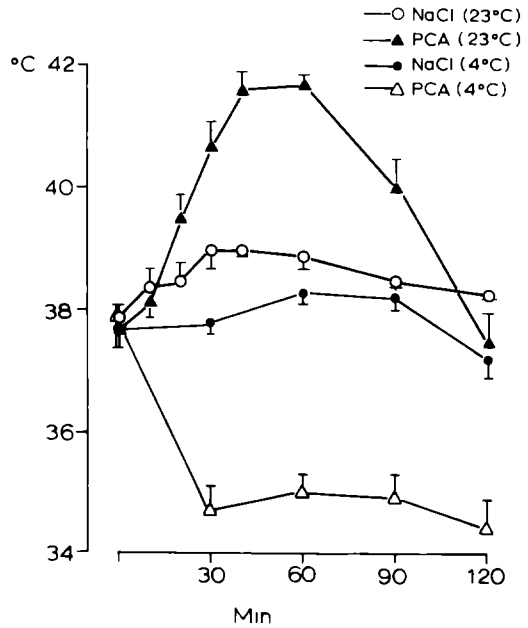

FIG 2 Body temperature after intraperitoneal injection of 25 mg/kg PCA in mice The animals were maintained in room temperature (23°C) or in cool temperature (4°C) as indicated

significant at all dose levels (5 mg/kg $F(1,16)=26\ 77$, $p<0\ 001$, 10 mg/kg $F(1,16)=39\ 76$, $p<0\ 001$, 40 mg/kg $F(1,16)=5\ 13$, $p<0\ 05$) Similarly there was also significant difference between time points at all dose levels (5 mg/kg $F(3,16)=19\ 74$, $p<0\ 0001$, 10 mg/kg $F(3,16)=13\ 14$, $p<0\ 0002$, 40 mg/kg $F(3,16)=24\ 91$, $p<0\ 0001$) No significant interaction was present between time points and structures at the 5 mg/kg dose, $F(3,16)<1$ There were, however, significant interactions at the 10 mg/kg dose, $F(3,16)=7\ 65$, $p<0\ 0025$, and the 40 mg/kg dose, $F(3,16)=4\ 82$, $p<0\ 02$

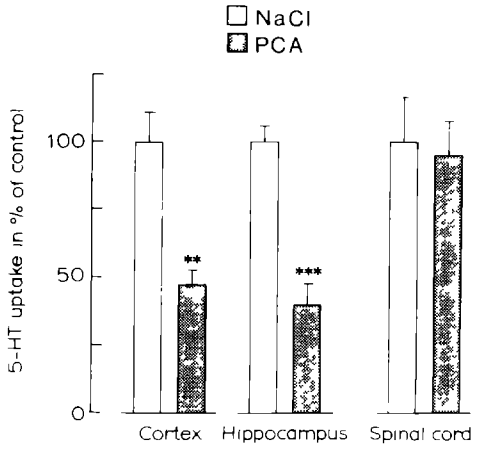

FIG 3 Uptake of ^{14}C-5-HT into cortical, hippocampal and spinal synaptosomes from PCA treated mice (40 mg/kg intraperitoneally on two consecutive days) Measurements were carried out on the 9th day after the last injection of PCA Mean±S E M of controls (n=6–8 for each group) Significant differences between vehicle and PCA treated groups are indicated by **($p<0$ 01) and ***($p<0$ 001) (Student's t-test)

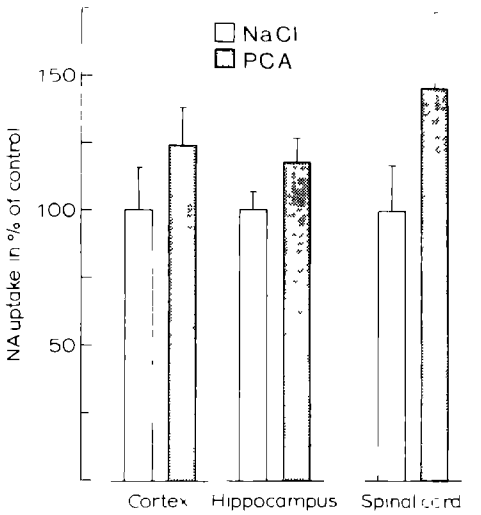

FIG 4 Uptake of ^{3}H-noradrenaline into cortical, hippocampal and spinal synaptosomes from PCA treated mice (40 mg/kg intraperitoneally on two consecutive days) Measurements were carried out on the 9th day after the first injection of PCA Mean±S E M of controls (n=7–9 for each group)

The concentrations obtained from the pons/medulla oblongata regions tended to fall between the values from the other structures

Changes in Body Temperature After a Single Dose of PCA

Twenty-five mg/kg PCA induced severe hyperthermia in the mice when tested at 23°C (Fig 2) The temperature reached a maximum of about 42°C after 40–60 min and returned to control level within 120 min of injection In the cold environment (4°C) 25 mg/kg PCA induced lower temperature than in the appropriate controls, the duration was more than 120 min Analyses of the results (ANOVA, 2 ambient temperature conditions by 2 drug treatment conditions, with 5 repeated measures of the body temperature) showed an overall effect of ambient temperature, $F(1,15)=266\ 87$, $p<0\ 0001$, and PCA treatment, $F(1,15)=23\ 61$, $p<0\ 0005$, with significant interaction, $F(1,15)=111\ 19$, $p<0\ 0001$ There was significant time effect, $F(4,60)=17\ 25$, $p<0\ 0001$, which showed interactions with ambient temperature, $F(4,60)=28\ 48$, $p<0\ 001$, PCA treatment, $F(4,60)=6\ 08$, $p<0\ 001$, and ambient temperature by PCA treatment, $F(4,60)=19\ 79$, $p<0\ 0001$

Biochemical Evaluation of the Lesions Effects of PCA on Synaptosomal Accumulation of ^{14}C-5-HT and ^{3}H-NA

Systemic injections of 40 mg/kg PCA on two consecutive days reduced the uptake of ^{14}C-5-HT in cortical, $t(13)=4\ 72$, $p<0\ 01$ and hippocampal, $t(13)=5\ 79$, $p<0\ 001$, synaptosomal preparations to between 40 and 50% of controls when measured on the 9th day after the last injection, while the uptake of 5-HT in the synaptosomes from the spinal cord was unaffected (Fig 3)

No statistically significant changes in uptake of ^{3}H-NA into cortical, hippocampal or spinal synaptosomal preparations were found, although minor increases in ^{3}H-NA uptake were present in all the structures tested (Fig 4)

Effect of PCA-Induced Serotonergic Lesions on Nociception and on the Antinociceptive Effect of Acutely Administered PCA

The results of the nociceptive testing are shown in Fig 5 Testing was performed 6–10 days after treatment with neurotoxic doses of PCA and 5 mg/kg of PCA was administered 30 min before testing The data for each test were analyzed seperately by ANOVA, using a two-pretreatments by two-test-treatments design

In the tail-flick test no significant main effects or interaction was present

In the hot-plate test there was no significant main effect of pretreatment, $F(1,36)=1\ 49$, $p>0\ 2$, whereas the test-treatment relation was significant, $F(1,36)=11\ 36$, $p<0\ 002$ Importantly, interaction between pretreatment and test-treatment was significant, $F(1,36)=4\ 56$, $p<0\ 05$ Further analysis was performed using the Scheffe's test The two groups that received saline as test treatment did not differ statistically PCA given as test-treatment significantly increased the response latencies in saline pretreated mice ($p<0\ 01$), but not in PCA pretreated animals

The data from the formalin test showed the same pattern as the data from the hot-plate test No significant main effect of pretreatment was present, $F(1,18)=1\ 90$, $p>0\ 15$ The effect of test-treatment was significant, $F(1,18)=4\ 55$, $p<0\ 05$, as was the interaction, $F(1,18)=7\ 72$, $p<0\ 02$ Scheffe's test showed no significant difference between the two groups that received saline at the day of testing, whereas significant effects of acutely administered PCA were present in the saline pretreated group ($p<0\ 025$) The score of the two groups that received acute PCA were also significantly different ($p<0\ 05$)

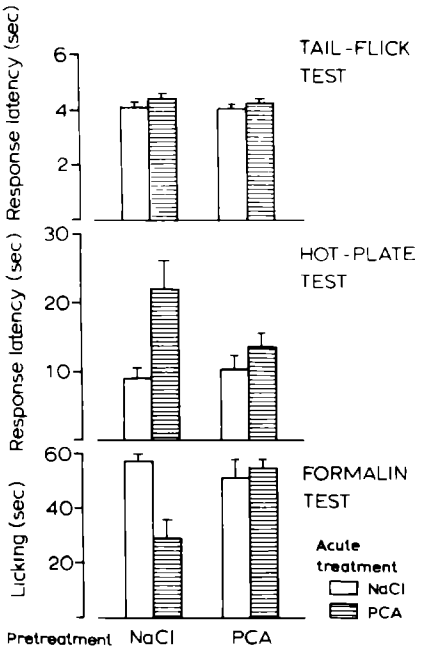

FIG 5 Effects of PCA-induced lesions (40 mg/kg intraperitoneally on two consecutive days) on nociception and on the antinociceptive effect of acutely administered PCA (5 mg/kg intraperitoneally 30 min before testing) in the tail-flick, hot-plate and formalin tests Testing took place 6–10 days after the first pretreatment with PCA Mean±S E M N=5–11 for each group Note that an increase in response latency in the tail-flick and hot-plate tests and a decrease in response in the formalin test imply antinociception

DISCUSSION

This study shows that when kept in a cool environment mice tolerate relatively high doses of PCA The compound induces substantial lesions of ascending serotonergic pathways as measured by uptake of ^{14}C-5-HT into synaptosomes prepared from frontal cortex and the hippocampus, but does not significantly affect the uptake of ^{14}C-5-HT in the spinal cord or uptake of ^{3}H-NA in any of the structures investigated

As previously shown in rats [20,21] acute administration of a lower dose of PCA acutely induced analgesia in the hot-plate test Significant analgesia was also found in the formalin test but not in the tail-flick test Pretreatment with PCA in neurotoxic doses prevented the analgesia induced by lower doses of PCA but did not alter the responsiveness to noxious stimulation in any of the tests in the absence of drugs

In the present study, a sublethal dose of PCA induced severe hyperthermia in the mice Furthermore, keeping the mice in a cool environment (4°C) reduced the body temperature and drastically reduced the lethality of PCA Thus, the present findings support the supposition that hyperthermia contributes to the acute toxicity of the compound [15] It should be noted that LD_{50} estimates after IP injection of PCA in mice vary from 12 [19] to 50 mg/kg [28] Factors besides ambient temperature contributing to the variations may include sex and body weight of the animals [15] and aggregation [5]

The lesions obtained in the present study were highly selective for supraspinal serotonergic structures as neither spinal serotonergic noradrenergic structures in the brain or spinal cord appeared to be affected These findings agree well with studies in the rat which also demonstrate a preferential long-term action on supraspinal serotonergic structures, although the efficacy of PCA appears to be higher in rats [16,26]

The 50–60% reduction in serotonergic accumulation into synaptosomes from cortex and hippocampus suggests that the lesions were similar in extent to those obtained in mice by Steranka and Sanders-Bush [29] using subcutaneously implanted osmotic mini-pumps which allowed continuous administration of PCA for 3 days In the latter study, 50–60% depletion of brain 5-HT was observed during the first 4 weeks after treatment whereas a substantial recovery took place between 4 and 8 weeks It seems likely that a similar recovery would take place after the lesions demonstrated in the present experiments

Significant reductions in 5-HT synaptosomal uptake or tissue levels do not always lead to a corresponding disruption of function For instance, recovery of function has been demonstrated in behavioral studies after lesioning of ascending as well as descending serotonergic pathways with dihydroxytryptamines, even when the biochemical parameters suggest persistent lesions of up to 90% of the serotonergic terminals [3, 8, 10] It is therefore significant that the relatively moderate reduction in ^{14}C-5-HT uptake induced by PCA treatment in the present study effectively prevented the analgesic effect of acutely administered PCA Thus, the method described provides a means for producing functional lesions of supraspinal serotonergic structures

Amphetamine is transported through the blood/brain barrier by means of a carrier common to aromatic amines [22,23] It is highly probable that PCA is transported via the same mechanism The carrier works bidirectionally according to the concentration gradient, and it has been reported that the half-life of PCA in the rat brain is much longer after intraperitoneal than after intracerebroventricular injection [27] The available information cannot decisively explain the regional differences in neurotoxic effect of PCA However, the fact that in the present study, lower concentrations of the compound were found in the spinal cord than in the cerebral cortex after all doses, suggests that variations in distribution of PCA in the CNS may at least be a contributing factor, although lower susceptibility to PCA of spinal tissue cannot be excluded The lower concentrations of PCA in the spinal cord may reflect poorer vascularization and blood supply in this region That the highest dose of PCA did not yield any differences in concentration between the structures at 30 min may be explained by a saturation of the transport system

The results obtained in the hot-plate and formalin assays agree with results in rats demonstrating analgesia in the paw-compression [11] and conventional hot-plate tests [20] as well as in the flinch-jump test and hot-plate test using slowly rising temperature [21] The finding that PCA-induced lesions eliminated the analgesic effect of subsequent lower doses supports previous conclusions that the effect involves supraspinal serotonergic structures [21] It should be noted, however, that a partial attenuation of PCA-induced analgesia also has been observed after selective lesioning of the descending serotonergic pathways [2] Possibly, noradrenergic and dopaminergic structures may also be involved in the effect of PCA since release of catecholamines has been reported after acute PCA treatment [26] Experiments in rats do not, however, support this hypothesis [21]

Thus, the results obtained with tests using complex, integrated behavioral responses to noxious stimulation un-

equivocally demonstrate analgesic activity of PCA. The absence of effect in the tail-flick test in the present study is therefore particularly interesting. In rats, either no [20] or weak biphasic effects of systemically administered PCA have been found in this test [21]. Furthermore, injection of PCA into the spinal subarachnoid space in mice produces analgesia in the tail-flick test, but not in a hot-plate test using slowly increasing temperature [4]. Taken together, these data suggest that lower doses of systemically injected PCA preferentially causes supraspinal release of 5-HT which may be insufficient to reduce the responsiveness in the tail-flick test. It appears likely that different serotonergic mechanisms regulate spinally integrated reflex responses and more complex behavioral responses to noxious stimuli.

In conclusion, this study describes a method for utilizing systemic administration of PCA as a tool for inducing selective lesions of supraspinal serotonergic structures in mice. The method produces functional lesions as demonstrated with behavioral tests. The data also confirms and extends previous data supporting a role for supraspinal serotonergic pathways in nociception.

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