Life Sciences, Vol. 27, pp. 1897-1904 Pergamon Press
Printed in the U.S.A.

EFFECTS OF CONTINUOUS GASTRIC INFUSION OF FOOD DYES ON DEVELOPING RAT PUPS

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(Received in final form September 11, 1980)

Summary

The consequences of chronic percutaneous gastric infusion of food dyes on the activity levels and shock avoidance performance of normal developing rats and those treated with 6-hydroxydopamine (6-OHDA) were investigated. All pups were artificially reared from four days of age and a liquid diet containing either food dyes (D) or no colorings (N) was infused into both 6-OHDA (T) and sham-treated pups (V). Pups receiving food dyes were significantly more active than comparable pups not receiving food dyes at all ages tested, an effect observed in both 6-OHDA and sham-treated pups. Pups treated with food dyes exhibited an impaired avoidance performance in a shuttle box at 28 days of age. VD and TN pups displayed an over 100% impairment in avoidance performance in comparison with VN pups. These results suggest that food dyes and/or their metabolites may exert deleterious effects on the behavioral repertoire of the developing rat pup.

While the consequences of the administration of a variety of drugs on the developing nervous system are well documented, new evidence suggests that many certified agents previously labelled "safe" or "harmless" may elicit subtle yet persistent effects in the developing organism. Recent public concern has focused on a possible link between the ingestion of artificial food additives and learning and behavioral problems in children (1). While several clinical studies have suggested that artificial food dyes may contribute to aberrant behavior in some children (2-6), few studies have investigated these effects in animals. Previous work in our laboratory has indicated that chronic administration of artificial food dyes to normal developing rat pups induced increases in activity and decrements in cognitive performance (7). In this earlier investigation, dyes were administered once daily as a bolus both to normal animals and littermates depleted of brain dopamine at five days of age with 6-hydroxydopamine (6-OHDA). Since 6-OHDA treatment has been suggested as a model for Minimal Brain Dysfunction (8), we have studied both normal and 6-OHDA treated pups in order to discern whether dyes might influence dopaminergic systems in the developing rat. In the present study, both normal and 6-OHDA pups were artificially reared beginning at four days of age. Rats were fed dyes through continuous gastric infusion of a liquid diet, rather than as a bolus. Our results suggest that these dyes elicit significant alterations in the behavior of both normal and 6-OHDA treated rat pups.

0024-3205/80/461897-08$02.00/0

Materials and Methods

Animals

Sprague-Dawley rat pups with mothers were obtained from Charles River, Inc., Wilmington, MA at 24 (± 12) hours of age and were individually housed in clean plastic cages (30x32x10 cm) with sawdust bedding. Mothers and pups were housed under flourescent lighting conditions with a 12 hour light-dark cycle commencing at 7:00 a.m. and 7:00 p.m., respectively at a temperature of 21°C. Purina Lab Chow and tap water were available to the dam ad libitum. These experiments included approximately equal numbers of male and female rats.

Subject Designation

Pups were pooled and numbered by toe punch at 4 days of age and were randomized as experimental or control according to a table of random numbers. Pups were assigned to one of 4 experimental groups: VD-sham treated pups fed food dyes (7 pups); VN-sham treated pups not fed food colorings (12 pups); TD-6-OHDA treated pups fed food dyes (6 pups); TN-6-OHDA treated pups not receiving dyes (12 pups).

Cannula Implantation

A permanent intragastric cannula was implanted in four day old rats using the method developed by Hall (9-11). The pups were lightly anesthetized with ether and a 6 cm length of piano wire (0.255 mm in diameter) was passed down the pup's throat and into the stomach through a lubricated sheath of silastic tubing. The wire was carefully passed through the left flank of the animal and was clamped outside. After removal of the silastic, the cannula was attached to the trailing end of the wire and was pulled through into the stomach. The cannula was constructed from a 17 cm length of PE-10 tubing, flared at one end so as to hold a polyethylene disc-parachute which would lodge and seal the cannula in the stomach. The cannula was secured at the fistula and in the neck flap with washers made from small lengths of PE-50 tubing.

Artificial Rearing and Dye Administration

Following intubation, the rat pups were housed individually in plastic cups, counter-weighted at the bottom with steel cylinders, which floated in temperature-controlled water baths. During the following two weeks, the bath temperature was slowly decreased from 37°C to 25°C on day nineteen. Sawdust bedding was changed every two to four days.

The pups were continuously fed a formula delivered from syringes driven by specifically modified Harvard Infusion Pumps, except for one hour periods each when pups were disconnected from the pump to facilitate formula changes and behavioral testing. The syringes were attached to the cannulas via a lead length of PE-100 and PE-50 tubing, the PE-50 end of which fit snugly around the end of the PE-10 cannula. Each day fresh formula was added and the PE-10 cannulas were flushed with saline. The liquid formula was based on Esbilac (Bordon), and was supplemented with l-methionine, l-tryptophan, B vitamins (Berroca-C, Roche), a vitamin mix (ICN), and a mineral mix (12). A mix of food dyes (prepared by H. Kohnstamm) was added where appropriate to the formula in order to deliver a daily dose of one milligram per kilogram. The mix contained eight dyes in varying quantities according to their average percent intake by humans: FD&C Blue No. 1, 3.12%; FD&C Blue No. 2, 1.7%; FD&C Green No. 3, 0.13%; FD&C Red No. 3, 6.0%; FD&C Red No. 40, 38.71%; FD&C Yellow No. 5, 26.91%; FD&C Yellow No. 6, 22.74%; and Orange B, 0.54%.

Weaning

At nineteen days of age, the cannulas were clipped off at the skin, and the rats were housed individually in clear plastic cages. The rats were fed a liquid diet of Sustacal (Mead-Johnson) supplemented as above, with food dyes added as appropriate. Amounts imbibed were recorded daily and no significant differences were seen among any of the groups.

Dopamine Depletion

6-hydroxydopamine HBr was purchased from Regis Chemical Company. Five day old rat pups were pretreated with desmethylimipramine (20 mg/kg intraperitoneally) followed one hour later by the intracisternal administration of 25 µl of 6-OHDA (100 µg per 25 µl, calculated as free base). Sham controls received 0.9 percent saline intraperitoneally followed one hour later by intracisternal administration of vehicle solution (0.9 percent saline plus 0.4 mg of l-ascorbic acid per milliliter).

Activity Measurement

Activity was determined at 12, 15, 19, and 26 days of age and measurements were always performed between 1200 and 1600 hours to minimize the variations due to circadian periodicity. Rat pups were randomly assigned to one of nine plastic cages (20x15x15 cm) on the floor of a sound-proof, temperature-controlled room. The activity of the pups was videotaped for one hour under infrared lighting. Scoring of activity was accomplished by playing the tape back at a speed equivalent to six times real time, and total activity in each rat was determined for alternate five minute periods throughout the 60 minute observation period. We thus had available for analysis six separate measurements of activity for each animal. The mean of these six determinations was then used to obtain the mean activity for the observation period. Activity was scored by the same observer in a blind fashion. An animal was considered to exhibit activity if any movement of any kind was observed (13).

Avoidance Learning

Avoidance learning in a shuttle box was determined at 28 days of age. The shuttle box was constructed of opaque Plexiglas with a floor of stainless steel rods and consisted of two compartments separated by a 5 cm hurdle, each compartment 20x14x17 cm high. Each animal was allowed 5 seconds following an initiating bell to avoid a 2.5 mA shock. If an animal failed to escape from the shock compartment in less than 30 seconds, he was manually placed in the free box. The number of avoidances over twenty trials was determined for each animal and the results were grouped into 5 blocks of 4 trials.

Determination of Catecholamines

Rats were sacrificed at 30 days of age between 0900 and 1200 hours in order to minimize brain catecholamine variation due to normal circadian periodicity. Brains were removed and frozen on dry ice within one minute after death. Frozen brains were stored at -70°C and biochemical determinations were performed within 2-3 weeks of sacrifice. Dopamine and norepinephrine were analyzed by flourometric techniques modified after procedures described previously by Roth and Stone and Boadle-Biber et al (14, 15).

Analysis of Data

Main effects and interactions in activity data were analyzed through a four-way analysis of variance. Main effects and interactions in shuttle box

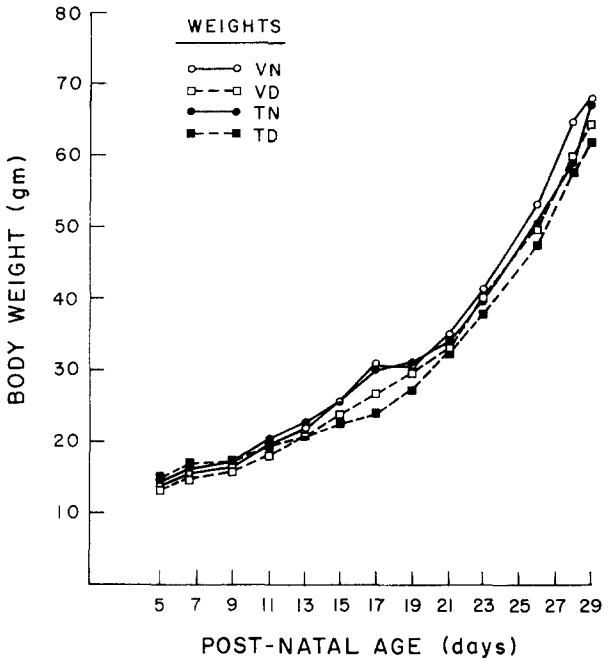
data were analyzed via a three-way analysis of variance. Post hoc examination of significant interactions was conducted with Scheffe's Test.

Results

Animal Growth Rates

Weights of all animals were recorded daily and no significant differences were detected among any of the four experimental groups (Fig. 1). The artificially reared pups showed slightly lowered body weights compared to mother-reared pups but other physical parameters such as eye-opening and hair development were normal.

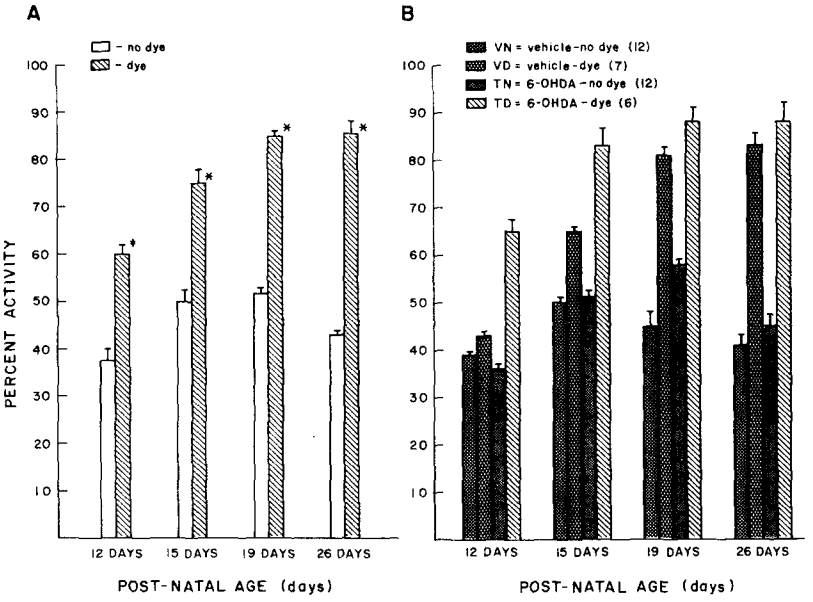
FIG. 1



Activity

Activity levels over all experimental groups increased from 45.6% at 12 days of age to 62.7% at 15 days of age, 68.2% at 19 days, and 64.2% at 26 days ($F(3,102)=11.28$, $p<0.001$). Rats receiving food dyes were significantly more active than their no-dye counterparts, averaging 74.7% overall activity in comparison with 45.6% for no-dye pups ($F(1,34)=32.90$, $p<0.001$). A significant age x food dye interaction emerged ($F(3,102)=3.60$, $p<0.025$); food dye pups were significantly more active than their no-dye counterparts at all four ages (Fig. 2a). Thus, while the activity of no-dye pups peaked at 19 days of age, the activity of food dye pups continued to increase throughout the first month of postnatal life. In our previous study, we found increased activity in only sham-treated pups. In the present study, both vehicle and 6-OHDA pups administered food dyes displayed comparable increases in activity. Figure 2b shows that TD pups displayed much greater increases in activity than VD pups at 12 and 15 days of age. However, no significant 6-OHDA x food dye or age x 6-OHDA x food dye interactions were detected.

FIG. 2



Dye effects on activity. a) Activity of dye and no-dye pups at each age; =$p<0.005$, *=$p<0.001$. b) Activity of the four treatment groups at each age.

In marked contrast with our previous studies, we found no significant increase in the activity of 6-OHDA pups as a group compared with their sham-treated counterparts. Nevertheless, as in our previous work, we detected a main effect of habituation ($F(5,170)=15.95$, $p<0.001$), indicating a general decline in activity during the testing period. In addition, a significant interaction between habituation and 6-OHDA emerged ($F(5,170)=2.88$, $p<0.025$) indicating that, as in our past investigations, while vehicle pups habituate to the testing environment, 6-OHDA pups displayed significantly impaired adaptability, (Goldenring and Shaywitz, manuscript in preparation).

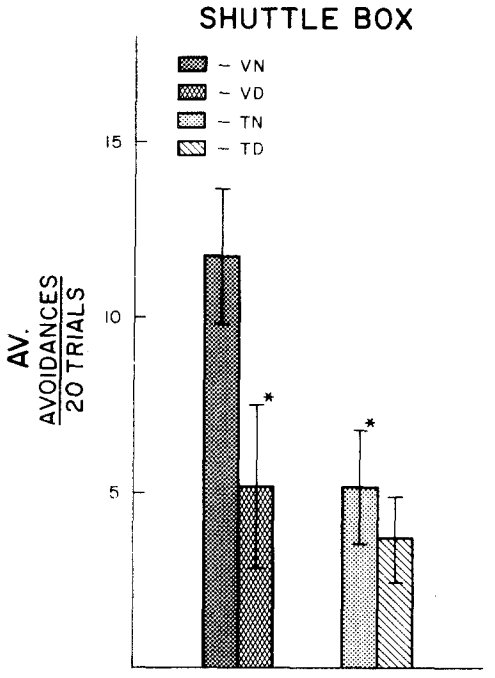
Avoidance Learning

Food dyes significantly disrupted avoidance performance in sham-treated pups ($F(1,34)=6.42$, $p<0.025$), who displayed a 128% greater failure rate when fed food colorings (Fig. 3). The performance of 6-OHDA treated pups was not affected by food dyes, perhaps on account of a ceiling effect. As in previous studies, 6-OHDA treated pups showed significantly impaired avoidance performance compared with sham-treated pups ($F(1,34)=5.75$, $p<0.025$).

Brain Catecholamines

Concentrations of brain dopamine were reduced to 25% of controls ($p<0.001$, t-test) in pups treated with 6-OHDA. Norepinephrine concentrations in 6-OHDA pups were not significantly different from controls ($p>0.05$, t-test). No effects of food dye administration on whole brain catecholamine concentrations were observed (data not shown).

FIG. 3



Food dye effects on avoidance performance. *=$p<0.025$ compared with VN pups.

Discussion

Although laborious and technically difficult, the artificial rearing procedure used in this study circumvented many difficulties frequently encountered in toxicological studies on developing animals. The artificial rearing procedure facilitated the direct delivery of an exact dosage of 1 mg/kg/day. Sobotka et al fed FD&C Yellow No. 5 to the lactating mothers of developing rats and found no significant behavioral alterations (16). However, such a protocol does not allow accurate estimate of the concentration of tartrazine actually fed to the pups via maternal milk. In fact, since Honohan et al and others (17-19) have found the Yellow No. 5 is fully metabolized in the intestines of adult rats, it is probable that maternal milk could contain only metabolites of the dye and not the actual dye itself.

Such studies as those detailed here must determine a physiologically appropriate dosage for investigation. Human studies on the effects of food dyes depend upon an extrapolation from per capita consumption, but precise amounts of dyes consumed have never been established (20). We have utilized such an extrapolation to calculate an overall dosage of 1 mg/kg/day which reflects the average human intake of the food dyes involved. However, children may well ingest much more than this average dose since candy, sodas, and other "snack" foods on the whole tend to contain higher amounts of food dyes. For example, four fluid ounces of Tang contain 3.78 mg of Yellow No. 5 (21).

The results of this investigation and our previous work indicate that food dyes have significant deleterious effects on the behavior of developing rats. In contrast with our earlier study on mother-reared rat pups (7), in the present investigation 6-OHDA treated pups also showed a marked increase in activity in response to food dyes. Our ability to observe this effect seems to reflect a fortuitous decrease in the activity of artificially reared 6-OHDA pups in comparison with their mother-reared counterparts in previous studies (Goldenring and Shaywitz, manuscript in preparation). However, as in previous studies 6-OHDA pups did display an inability to habituate to the test environment, perhaps a more sensitive indicator of the behavioral impact of dopamine depletion. Also as reported previously, dopamine-depleted pups continued to exhibit significant disruption in shuttle box performance when compared with their littermate controls (8).

While the mechanism of these profound food dye effects is not clear, our hypothesis suggest that dyes or their metabolites in some way exert a direct influence on the brain. The apparently increased effects seen in 6-OHDA pups at 12 and 15 days of age may suggest some involvement of monoaminergic systems in the central nervous system. However, expected levels of whole brain dopamine and norepinephrine were not altered by food dye administration.

Since eight different dyes were incorporated into the dye mixture in varying amounts, it is impossible to determine which of the colorings or combinations may have been responsible for the aberrant behavioral effects. Nevertheless, it is reasonable to expect that the noxious agent was one of the four major components of the mix: Yellow No. 5, Yellow No. 6, Red No. 40 or Red No. 3. Yellow No. 5 and Yellow No. 6 have been known to produce allergic reactions (22) and furthermore, allergy to these dyes seems to correlate to a great extent with allergy to aspirin (23). Red No. 3 has recently been shown to alter the electrophysiological properties of invertebrate neurons (24) as well as to modify transmission at the frog neuromuscular junction (25). In addition, Red No. 3 has been shown to decrease neurotransmitter reuptake in homogenates and synaptosomes (26, 27). All of these dyes have been implicated as possible causative or aggravating agents in hyperactivity and learning disabilities seen in children (1-6).

It is also possible that the observed reactions are due to metabolic products of the dyes rather than the parent compounds themselves. The first three dyes noted above (i.e. excluding Red No. 3) are azo dyes which are cleaved in the intestines with very high efficiency (16-18). The metabolites after cleavage are all aromatic or polyaromatic sulfonic acid derivatives, most of which are highly charged species which one might not expect to cross the blood-barrier. Unfortunately, the only investigation of the destination of azo dye metabolites studied only absorption in adult rats and did not assay brain tissue. However, it is important to note that in that study, 37.4% of sulfanilic acid, a major dye cleavage product, was not excreted by adult rats 24 hours after its initial administration (16). Certainly then much of the dyes' metabolites is absorbed from the intestines. Furthermore, tracer studies now under way in our laboratory indicate that sulfanilic acid does cross the blood-brain barrier (Goldenring and Shaywitz, manuscript in preparation). Future studies will be needed to precisely define the destination and actions of food dyes and their metabolites in the developing organism.

Acknowledgements

This study was supported by PHS Grants NS 12384, AA 03599, HD 03008, NIMH Grant #1 P 50 MM 30929, The Thrasher Research Foundation and the Nutrition Foundation.

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