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Brain Dopamine Response in Isolated 10-Day-Old Rats: Assessment Using D_2 Binding and Dopamine Turnover

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KEHOE, P., K. CLASH, K. SKIPSEY AND W. J. SHOEMAKER. *Brain dopamine response in isolated 10-day-old rats: Assessment using D_2 binding and dopamine turnover.* PHARMACOL BIOCHEM BEHAV **53**(1) 41-49, 1996.—A single 5-min isolation from the nest, dam, and siblings in 10-day-old rat pups was investigated for its effect on brain dopamine systems. The release of dopamine in innervated brain regions was measured in separate studies using in vivo ligand binding of ^{3}H-raclopride, ex vivo binding using ^{3}H-raclopride, and neurochemical measurement of the dopamine turnover using levels of DOPAC and dopamine. In addition, in vitro homogenate binding was performed to determine baseline B_{max} and K_d values for ^{3}H-raclopride binding sites across treatments. Isolation for 5 min in a "novel" environment resulted in decreased ^{3}H-raclopride binding in striatum and septum as determined by both in vivo and ex vivo binding, as well as increased dopamine turnover. There was no difference in B_{max} and K_d values for ^{3}H-raclopride in these brain regions after the 5-min isolation, indicating that the binding decreases were due to an increase of available dopamine, presumably from terminal release. The convergence of results from three different techniques supports the interpretation that dopamine is released during the 5-min isolation in both brain regions.

Dopamine Turnover Rat pup Stress Isolation Development In vivo binding Ex vivo binding In vitro binding DOPAC

SEVERAL studies have demonstrated that stressful experiences result in neurotransmitter changes in the brain. Among the neurotransmitters reported are norepinephrine (2,14,30, 34,43), serotonin (2,20), and dopamine (1). These studies use tail-pinch, tail-shock, or restraint stress in adult animals.

Several brain neuropeptides have also been demonstrated to respond following various stressors. Using in situ hybridization, mRNA's for neurotensin and enkephalin were measured in the hypothalamus following stressors such as ether, immobilization, cold, and swimming (6). Corticotropin-releasing factor (CRF) has also been implicated as an important aspect of the brain's response to stress (41). Some of the transmitter responses to stress have been linked to immediate early gene activation. Specifically, c-fos has been shown to be increased in the lateral septal nucleus and paraventricular nucleus (PVN) following swim stress (12) and in the PVN following immobilization stress (7). In this latter study, several of the neurons positive for c-fos were also positive for CRF.

In agreement with the adult studies, our prior work with postnatal day 10 (PN 10) rat pups indicated that β-endorphin and enkephalin in the brain stem and periaqueductal grey regions are activated by isolation, a neonatal stressor (40). In that prior study, release of the opioid peptides was seen at both 5 and 30 min following isolation. Similarly, using in vivo binding, Insel (19) found an activation of GABA systems in the neocortex, hippocampus, and inferior culliculi of rat pups after social isolation. In another neonatal study, Keller et al. (28) showed that under certain circumstances, tail-pinch can activate dopamine release in the ventral striatum. In neonatal guinea pigs, isolation produces increased dopamine turnover in the septal region and anterior hypothalamus (15,42).

The present study reports the effects of a single 5-min isola-

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tion stress on the activation of dopamine in the striatum and septal regions of the brain in 10-day-old rat pups. The striatum receives a large dopaminergic innervation from the nigrostriatal system and has been shown to respond to stresses by releasing dopamine (1,22). The septum receives a dopamine innervation from the mesolimbic system and has been shown to respond to immobilization stress (37,42). Furthermore, the study used several different methods of assessing dopamine activation in an attempt to obtain converging evidence.

Within this study is a comparison of two different isolation conditions, because our earlier studies demonstrate that specific stimuli present during the isolation period result in differential neurotransmitter activation that correlates with different behaviors and pharmacologic responsiveness (40). The isolation experience can be considered stressful because it activates transmitter release, crying behavior, analgesia, and the hypothalamic–pituitary–adrenal axis in a number of mammalian species (16,17,18,25,27,32,35,42). In primates, separation from the mother or other caregivers can affect the activity of central monoaminergic systems (8,31). Tamborski et al. (42) demonstrated that central dopamine turnover in guinea pig pups during separation from their mothers closely parallels findings in separated monkeys. In rats, repeated isolation experiences can lead to hyper-responsivity to psychomotor stimulants (i.e., sensitized behavior) (5,46). One of the hallmarks of sensitized behavior is involvement of the ascending dopamine systems. Thus, activation of the dopamine system by a single acute episode of isolation could be a part of the initiation of sensitization (21).

METHODS

Subjects

Sprague–Dawley female rats (Charles River, Wilmington, MA) were mated in the Trinity College psychobiology animal colony; their litters served as subjects. Animals were housed in plastic tubs with wood chip bedding and stainless-steel wire lids. Purina Rat Chow (Frenchtown, NJ) and water were available ad lib. Lights in the colony were on from 0700–1900 h. Room temperature was maintained between 23 and 25°C. Newborn litters found until 1900 h were considered born on that day (day 0). Litters were culled to 12 with sexes balanced on day 1. The study employed a "within-litter" design; thus, two pups for each treatment for each sex were included in every litter.

Isolation Treatment

On postnatal day 10 (PN 10), the dam was removed from the nest, and the cage was placed on a heating pad for warmth. The temperature of the nest was maintained at 32°C. When pups were removed for treatment, untreated 10-day-old pups were placed in the nest to maintain at least eight pups in the huddle at all times. For one isolation condition, each pup was removed from the nest and placed in a cup (4 × 6 cm) containing 50 cc of fresh bedding (the same bedding used in the home cage) for 5 min in an environmental chamber maintained at 32°C. In another condition each pup was isolated in a small wooden box (20 × 20 cm) with no bedding for 5 min, also in an environmental chamber maintained at 32°C. The box had a screen bottom that was cleaned with water and dried between the use of animals. We characterized the condition in which the pups were isolated in bedding as "familiar" because in our colony the pups were exposed to the odor and texture of fresh bedding during the first 10 postnatal days. The condition of isolating in the wooden box was termed "novel" because there were no objects to which the pup had been previously exposed. A third group of pups was removed from the nest in a cup and taken to the benchtop for decapitation. These pups were called "nest" controls and received no isolation treatment. (For in vivo binding, nest controls were injected with radiolabeled tracer and placed in a huddle before decapitation.) However, note that all treatment groups were "handled" in an equivalent manner.

In Vivo Binding

Postisolation treatment for in vivo binding. Following one of the isolation treatments or additional time in the nest, the animals were injected intraperitoneally with 1 μCi (0.05 ml) of ^{3}H-raclopride, a ligand that binds with high affinity to the dopamine-D_2 receptor site (11). In addition, raclopride binding has been studied both in vitro and in vivo; under in vivo conditions, raclopride binding is saturable and reversible, and has low nonspecific binding (29). Following the injection, the pup was huddled in a heated chamber with other pups for 20 min. The amount of tracer injected and the time interval between injection and decapitation (i.e., incubation period) were chosen following a pilot study in which several tracer amounts and time periods were examined. Immediately following the termination of the incubation period, the pup was decapitated and the brain rapidly removed from the cranium. The striatum, septum, and cerebellum were dissected from the brain according to the 10-day postnatal atlas of the developing rat brain by Sherwood and Timiras (39).

Binding assay. Each brain region dissected from a single animal was placed in a preweighed separate vial that contained 750 μl of Solvable (DuPont Biotechnology Systems, Boston, MA), a tissue dissolver. The weight of the tissue was recorded and the vial was warmed overnight at 53°C. The following day each vial received 15 ml of Formula 989 Fluor Cocktail (DuPont) and was placed in an LBK 1218 Rackbeta scintillation counter (Wallac, Inc., Gaithersburg, MD). The resultant counts were converted to disintegrations per minute (dpm) per milligram tissue using a quench correction curve and efficiency measures. Prior studies found that additional injections of "cold" (nonradioactive) raclopride to the animals, compared with those receiving saline injections, decreased counts in striatum and septum but not cerebellum, indicating the nonspecific nature of the cerebellar counts [see also (36)]. This allows for the correction of individual animal differences in injected amount, blood flow, or other factors that could affect the distribution of radioligand to the brain. Results were corrected for counting efficiency and expressed as dpm per milligram of tissue.

Ex Vivo Binding

Postisolation treatment for ex vivo binding. Pups were decapitated immediately following isolation treatment or being taken from the nest. The brain was removed from the cranium and placed in phosphate-buffered saline (PBS) at 4°C for 20 min. For the striatum, striatal tissue from both hemispheres was removed from each brain, placed in a McIlwain Tissue Chopper (Brinkman Instruments, Westbury, NY), and cut into 300-μm coronal slices according to the procedures of Wilkinson et al. (44). For the septum, one dissected piece from the midline was removed and similarly sliced in the tissue chopper. The sliced tissue was separated in PBS (4°C) with fine forceps on a Petri dish coated with Sylgard (Dow Corning, Corning, NY). Punches, 2 mm in diameter, were made

from the slices and one punch transferred to each individual well of a microwell plate. Each assay run was conducted with 2 litters of 12 pups each (total of 12 males and 12 females). The 24 pups were divided into three groups of eight pups (four males and four females) each for the three treatments (nest, familiar, and novel). This procedure was repeated 11 times.

Binding assay. Each well contained 500 μl of cold PBS buffer. Three wells were used for nonspecific binding (NSB) by incubation in the presence of 20 μl of 10^{-4} M nonradioactive raclopride. After the 15-min preincubation period, both total and nonspecific specimens were incubated at 30°C for 1 h in the presence of 20 μl of ^{3}H-raclopride (2.5 nM). These parameters were chosen following pilot studies using a range of ^{3}H-raclopride concentrations (0.5–10 nM); an optimal ratio of specific to nonspecific binding was obtained at 2.5 nM of the ligand.

Following incubation, the trays containing the tissue were placed on ice. We removed 50-μl aliquots of incubation buffer from each of the total binding wells and placed them in scintillation vials to determine the concentration of free radiolabeled ligand. The tissue punches were then washed twice with 500 μl PBS (4°C) using a jet pipette. Buffer was removed using glass pipettes, and tissue punches were then transferred with glass fiber filter paper to labeled scintillation vials. We added 5 ml of Formula-963 Fluor Cocktail to each vial. The vials were shaken and placed in the dark overnight. Samples were then counted in an LBK 1218 Rackbeta scintillation counter to determine total and nonspecific binding. Total binding was defined as the binding of the radiolabeled ligand in the absence of nonlabeled (cold) ligand and was determined using four tissue punches.

In Vitro Binding

Postisolation treatment for in vitro binding. Immediately following the isolation or nest period, the rat pups were rapidly decapitated and their brains removed and dissected on ice for striatal and septal tissue. The tissue was stored frozen at −70°C.

Binding assay. Tissue was pooled (six for striatum and nine for septum) and prepared for homogenization by thawing on ice and cutting with a cold, sharp razor blade. Sliced tissues were placed in 50 vol. of Tris-HCl buffer (pH 7.4) on ice and homogenized with a high-speed Tissuemizer (Tekmar, Cincinnati, OH) for five strokes. After centrifugation at 20,000 × *g* for 10 min at 4°C, the pellet was washed, recentrifuged, and resuspended in 10 ml of Tris-HCl buffer containing 120 mM NaCl and 5 mM KCl. The reaction mixture contained 200 μl of the membrane preparation, 200 μl of Tris-HCl buffer, and one of 12 concentrations of cold raclopride (range 0.5×10^{-15} to 5×10^{-10} vol. = 50 μl). The incubation was started by the addition of 50 μl of ^{3}H-raclopride at a concentration of 1 nM and continued for 45 min at 25°C. This concentration of labeled ligand was chosen because it is close to the K_d value, and Kohler et al. (29) found single-site binding of ^{3}H-raclopride in a range of 0.25–6 nM in saturation binding. In addition, Dewar et al. (11) found only one binding site in rat striatum using heterologous displacement binding. Tissue-bound ligand was separated from unbound ligand by rapid filtration over Whatman GF/C glass fiber filters (Whatman Int'l Ltd., Maidstone, Kent, UK), followed by two washes of 5 ml cold Tris-HCl buffer. Dried filters were placed in vials with 3 ml of Picofluor (Packard Instruments Co., Meriden, CT) and counted in a Beckman LS3801 scintillation counter. Raw data were entered and competition curves generated using a program written in the laboratory (10). Data were further analyzed and K_d and B_{max} values calculated by LIGAND (33) run in a VAX cluster (VAX Scafit version 2.5, Digital Equipment Corp., Maynard, MA). Protein determinations of a membrane preparation aliquot were performed using the Pierce BCA protein assay (Pierce Co., Rockford, IL).

Neurochemical Assay for DOPAC and Dopamine

Postisolation treatment for neurochemical assays. Pups were decapitated immediately following the isolation treatment or being taken from the nest; their brains were rapidly removed, and the septum and striatum dissected, weighed, and stored at −70°C.

HPLC assay for DOPAC and dopamine. In preparation for the HPLC assay, the tissue was defrosted with the addition of 250 μl of cold 0.1 M perchloric acid ($HClO_4$) and 250 μl of 1×10^{-6} dihydroxybenzylamine (DHBA) as the internal standard. Sonication for 20 s (Vibracell, Sonics and Materials, Danbury, CT) was followed by centrifugation for 10 min (model 235B; Fisher Micro-Centrifuge, Fisher Scientific, Pittsburgh, PA). The supernatants were separated on a Hypersil ODS (ESA, Chelmsford, MA) 3μm column (100 × 4.6 mm) with a mobile phase of 0.1 M sodium phosphate, 135 mg/l sodium octyl sulfate, 50 mg/l EDTA, and 10 ml/l acetone (pH 3.6–3.7) at a flow rate of 1.25 ml/min. Detection was analyzed by an EG&G (Princeton, NJ) (model 400) electrochemical detector.

Statistical Analyses

For each of the assays, we analyzed data with 3 (condition = nest, familiar, novel) × 2 (gender) analysis of variance (ANOVA). Because there were no significant gender differences, the data for males and females were combined. Pairwise comparisons with Tukey's honestly significant difference (HSD) test were done on significant ANOVAs.

RESULTS

Effect of Isolation Conditions on In Vivo Specific Binding of ^{3}H-Raclopride in Striatal and Septal Region of 10-Day-Old Rat Pups

The effect of a 5-min isolation period on the specific binding of ^{3}H-raclopride is shown in Fig. 1a for the corpus striatum, and Fig. 1b for the septal region. In each animal, nonspecific binding was determined by using radioactive levels in the cerebellum. ANOVA indicated that the effect of isolation condition was highly significant in the striatum [$F(2, 42) = 5.2$, $p < 0.01$], and that it was the novel isolation condition in which the reduction in dpm per milligram was significantly less than the nest condition (Tukey HSD, $p < 0.01$). We interpreted the reduction in specific ligand binding level as an increase in receptor occupancy by endogenous dopamine, because the total number of receptors in this region did not change (see subsequent description). A similar finding was observed in the septal region of the same animals [$F(2, 42) = 5.7$, $p < 0.01$]. In the septum, the novel condition was significantly different from the nest condition, (Tukey HSD, $p < 0.01$) and from the familiar condition ($p < 0.05$).

Effect of Isolation Conditions on Ex Vivo Specific Binding of ^{3}H-Raclopride in Striatum and Septum of 10-Day-Old Rat Pups

The effects of a 5-min isolation period on the specific binding of ^{3}H-raclopride using the ex vivo technique are shown in

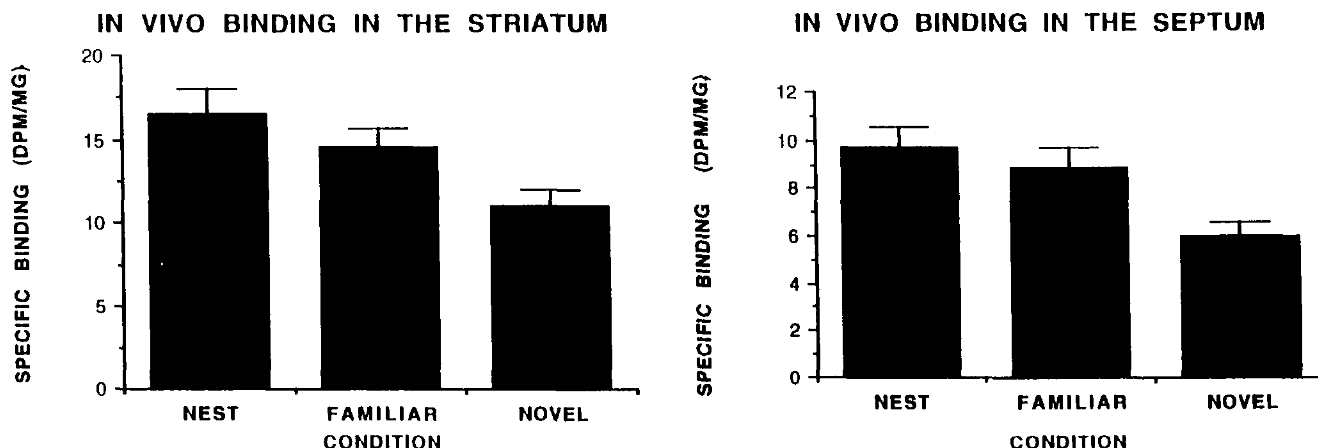

FIG. 1. (a) Specific binding of ^{3}H-raclopride in the striatum using the in vivo binding technique. Results are expressed as mean (±SEM) disintegrations per minute (dpm) per milligram of brain tissue after correction for nonspecific binding. At PN 10, pups were assigned to one of three conditions that involved remaining in the nest until the radioligand was injected, or isolation in a "familiar" or "novel" condition for 5 min before ^{3}H-raclopride administration. Results are the means of 15 for nest, 16 for familiar, and 14 for novel. Statistics are reported in Results. (b) Specific binding of ^{3}H-raclopride in the septum using the in vivo binding technique. Results are expressed as mean (±SEM) disintegrations per minute (dpm) per milligram of brain tissue after correction for nonspecific binding. At PN 10, pups were assigned to one of three conditions that involved remaining in the nest until the radioligand was injected, or isolation in a "familiar" or "novel" condition for 5 min before ^{3}H-raclopride administration. Results are the means of 15 for nest, 16 for familiar, and 14 for novel. Statistics are reported in Results.

Fig. 2a for the striatum, and Fig. 2b for the septum. The results were similar for both brain regions in that there was a significant effect of isolation [$F(2, 26) = 3.26$, $p < 0.05$ for the striatum and $F(2, 30) = 3.80$, $p < 0.03$ for the septum]. Furthermore, posthoc Tukey's HSD test revealed that the novel isolation treatment differed significantly from the nest condition in both striatum ($p < 0.04$) and septum ($p < 0.04$). These ex vivo binding results are remarkably similar to the in vivo results shown in Fig. 1 in that they both provide evidence that receptors were functionally less available to bind exogenous ligand during isolation (i.e., they had lower labeled ligand binding). Moreover, the comparison of the two isola-

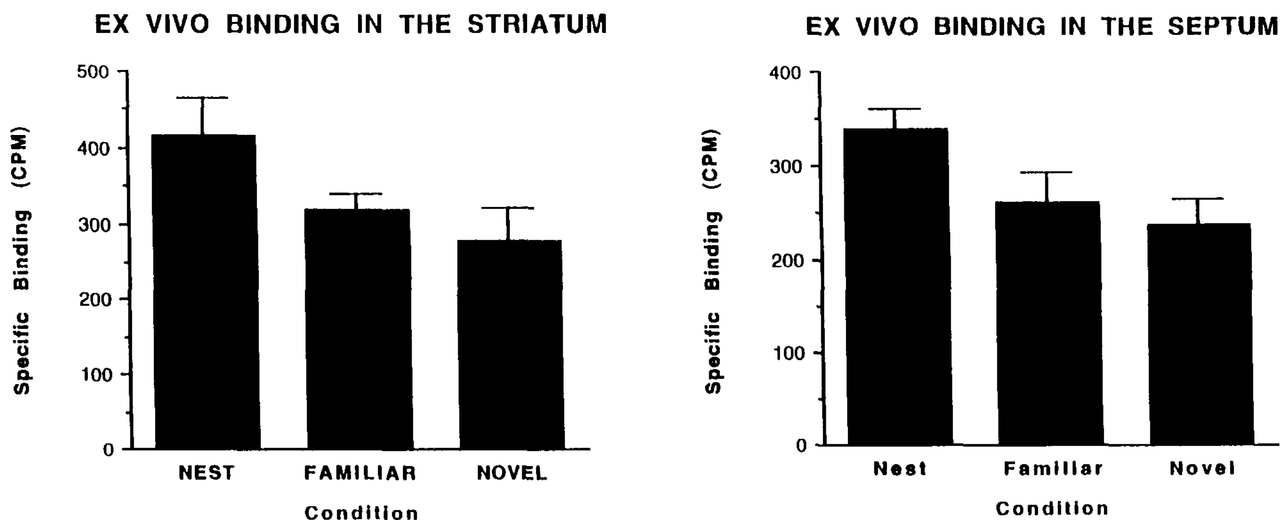

FIG. 2. (a) Specific binding of ^{3}H-raclopride in the striatum using the ex vivo binding technique. Results were derived by subtracting the mean CPM of the tissue punches designated as nonspecific binding from those designated as total. Pups were assigned one of three conditions: remaining in the nest, or isolation in a "familiar" or "novel" condition for 5 min before decapitation and brain removal. Results are the means (±SEM) of 11 assays for each treatment group. Statistical significance is discussed in Results. (b) Specific binding of ^{3}H-raclopride in the septum using the ex vivo binding technique. Results were derived by subtracting the mean CPM of the tissue punches designated as nonspecific binding from those designated as total. Pups were assigned one of three conditions: remaining in the nest, or isolation in a "familiar" or "novel" condition for 5 min before decapitation and brain removal. Results are the means (±SEM) of 11 assays for each treatment group. Statistical significance is discussed in Results.

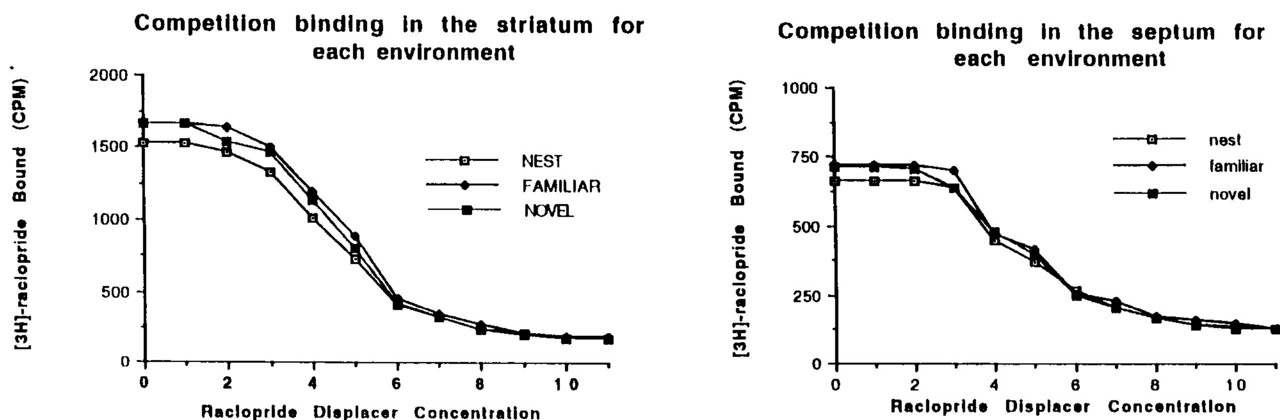

FIG. 3. (a) Displacement binding curves for ^{3}H-raclopride binding to homogenates of the striatum from pups of each of the three conditions: taken from the nest or isolated in "familiar" or "novel" conditions. Each assay for each treatment condition was performed four times, each time in triplicate. Incubation conditions were at 22°C for 90 min in the presence of 1 nM ^{3}H-raclopride and 12 concentrations of nonradioactive raclopride displacer. The concentration of "cold" raclopride in each of the 12 conditions is as follows: concentration indication 0 = 0.0 M; 1 = 5×10^{-15} M; 2 = 1×10^{-14} M; 3 = 5×10^{-14} M; 4 = 1×10^{-13} M; 5 = 5×10^{-13} M; 6 = 1×10^{-12} M; 7 = 5×10^{-12} M; 8 = 1×10^{-11} M; 9 = 5×10^{-11} M; 10 = 1×10^{-10} M; 11 = 5×10^{-10} M. The curves for each treatment best fit a one-site model as determined by LIGAND. Nonspecific binding is indicated by the plateau of binding at higher displacement concentrations. Nonspecific binding is factored into the determination of B_{max} and K_d (see Table 1). (b) Displacement binding curves for ^{3}H-raclopride binding to homogenates of the septal region from pups of each of the three conditions: taken from the nest or isolation in "familiar" or "novel" conditions. Each assay for each treatment condition was performed three times, each time in triplicate. Incubation conditions were at 22°C for 90 min in the presence of 1 nM ^{3}H-raclopride and 12 concentrations of nonradioactive raclopride displacer. The concentration of "cold" raclopride in each of the 12 conditions is as follows: concentration indication 0 = 0.0 M; 1 = 5×10^{-15} M; 2 = 1×10^{-14} M; 3 = 5×10^{-14} M; 4 = 1×10^{-13} M; 5 = 5×10^{-13} M; 6 = 1×10^{-12} M; 7 = 5×10^{-12} M; 8 = 1×10^{-11} M; 9 = 5×10^{-11} M; 10 = 1×10^{-10} M; 11 = 5×10^{-10} M. The curves for each treatment best fit a one-site model as determined by LIGAND. Nonspecific binding is indicated by the plateau of binding at higher displacement concentrations. Nonspecific binding is factored into the determination of B_{max} and K_d (see Table 2).

tion conditions indicates that isolation in a novel environment resulted in consistently lower receptor occupancy by the ligand, reaching statistical significance in each region using either technique. Isolation of pups under familiar conditions resulted in levels of occupancy intermediate between the nest and the novel condition.

Effect of Isolation Condition on In Vitro Ligand Binding in Homogenates of Striatum and Septum

Traditional in vitro homogenate binding was performed on brain regions of rat pups treated identically to those in the in vivo and ex vivo binding studies. Because the latter studies indicated differences in ^{3}H-raclopride binding related to isolation, it was important to determine whether these differences were due to changes in the absolute number of binding sites, changes in the affinity of the ligand for the binding site, or competition between released dopamine and the labeled ligand. Figure 3 graphically depicts the displacement binding isotherms from representative striatal (a) and septal (b) tissues for the three experimental conditions. Binding data from four to six determinations for each condition were subsequently transformed by LIGAND to produce best-fit data for the number of binding sites and determination of affinity (K_d) and capacity (B_{max}) (33). Each displacement curve best fit a single-site model for binding and yielded K_d and B_{max} estimates, as shown in Tables 1 and 2. These results, which are similar to previously published figures for adults (11), indicate that there are no differences in either capacity or affinity of the ^{3}H-raclopride binding sites as a result of the isolation conditions imposed on these animals. This result is expected, because the isolation was imposed just 5 min before sacrifice, and changes in receptor number may require a longer time period to be affected.

The in vitro homogenate binding results suggest that the changes seen in both in vivo and ex vivo ^{3}H-raclopride binding following isolation were not due to alteration in D_2 receptor characteristics but more likely the result of competition of the

TABLE 1

EFFECT OF CONDITION ON B_{max} AND K_d OF ^{3}H-RACLOPRIDE BINDING IN THE STRIATUM

Condition	B_{max} (fmol/mg Protein)	K_d (nM)
Nest	268.9 ± 75.17	0.364 ± 0.23
Familiar	253.8 ± 63.83	0.391 ± 0.18
Novel	282.8 ± 60.82	0.340 ± 0.62

TABLE 2

EFFECT OF CONDITION ON B_{max} AND K_d OF ^{3}H-RACLOPRIDE BINDING IN THE SEPTUM

Condition	B_{max} (fmol/mg Protein)	K_d (nM)
Nest	147.9 ± 38.33	0.281 ± 0.22
Familiar	157.3 ± 52.73	0.370 ± 0.36
Novel	141.0 ± 22.46	0.326 ± 0.30

labeled ligand with endogenous transmitter for the binding site.

Effect of Isolation Conditions on Levels of DOPAC and Dopamine in Septum and Striatum

The effects of a 5-min isolation period on the tissue levels of DOPAC and dopamine and DOPAC/dopamine ratio as determined by HPLC and electrochemical detection are shown in Fig. 4a for striatum and Fig. 4b for septum. In the striatum (Fig. 4a) there was a significant effect of treatment on the DOPAC/dopamine ratio [$F(2, 53) = 4.92$, $p < 0.01$], where the novel isolation condition was significantly increased over the nest control ($p < 0.03$, Tukey HSD post hoc test). In the septum (Fig. 4b), there was also a significant effect of treatment on DOPAC/dopamine ratio [$F(2, 49) = 3.63$, $p < 0.03$], where novel isolation was significantly increased over nest controls (Tukey HSD, $p < 0.03$). DOPAC, a major metabolite of dopamine, is produced in higher levels when dopamine is released from terminals (9,13,15). The ratio of DOPAC to dopamine in the tissue sample is often used as an indicator of dopamine turnover (15,42).

DISCUSSION

Isolation stress at PN 10 resulted in measurable involvement of the brain dopamine system using any of the three methods employed. In fact, there was a remarkable consistency in the direction and level achieved across methods when the results of all three methods are compared using percentage change (Table 3). The greatest change from the controls was in those pups isolated in novel circumstances. Those pups isolated in "familiar" environments produced some indication of dopamine involvement, but these did not reach statistical significance. This is similar to the results of Tamborski et al. (42), who found in neonatal guinea pigs that pups isolated in a novel environment with their mothers produced levels of dopamine metabolites that were intermediate between those pups remaining in the nest (controls) and those isolated alone in the novel environment. Presumably, the presence of the mother adds some "familiarity" to the novel environment.

In the studies reported here, the DOPAC/dopamine ratio was increased in the novel situation in both striatum and septal regions. This increase is interpreted to be an increased turnover of dopamine in the terminals of these regions. In this regard, our data differed from those of Tamborski et al. (42), who reported that their isolation produced significant effects in the septum, but not in the striatum. We cannot explain this discrepancy except to point out that their study was carried out on 15 day-old guinea pigs, a species more precocial than rats.

TABLE 3

COMPARISON OF PERCENTAGE CHANGE FROM THE NEST CONDITION OF ISOLATION TREATMENTS FOR EACH OF THREE METHODS OF ESTIMATING DOPAMINE RELEASE

	Familiar (%)	Novel (%)
In vivo binding		
Striatum	12	34
Septum	9	38
Ex vivo binding		
Striatum	28	47
Septum	23	31
Dopamine turnover		
Striatum	6	38
Septum	20	41

One of the advantages of using several techniques to assess the same end point is that artifacts associated with a single technique can be exposed or ruled out. With in vivo binding, for instance, it is possible that the stress associated with the 5-min isolation could affect the distribution of the radiolabeled raclopride through effects on vasoconstriction or other means. However, the method we employed measured the radiolabeled binding in the cerebellum of each animal to normalize the labeled counts in the other brain regions of that animal. Because there are extremely low levels of dopamine receptors in the cerebellum, changes in cerebellar counts resulting from nonspecific binding would control for differences in injected amount, effects of blood flow, or other factors. The results obtained from the ex vivo binding technique, in which the radioactive raclopride is added to the tissue after it is excised from the brain, agree very closely with the results obtained from the in vivo binding (Table 3).

In fact, the three techniques employed in the present study (in vivo binding, ex vivo binding, and neurochemical assay) gave results that indicated similar levels of dopamine activation following isolation (Table 3). The results using the ex vivo binding techniques gave somewhat greater percentage differences from controls, but the variance was not appreciably different. It may be useful to view the different techniques along a continuum of "biological intactness." The in vivo binding is carried out in an awake, behaving animal and could represent the most intact preparation. Of course, one must later obtain the tissue from the animal to assess the level of binding. In ex vivo binding, the tissue is removed from the animal initially, before binding is carried out. Although considerable efforts are made in the ex vivo technique to preserve function in the tissue punches (44), the loss of neural input from proximal sites in the brain may lessen the rating of intactness compared with in vivo binding. Incidentally, the greater control of the equilibrium binding process in ex vivo binding and the lack of interfering signals may contribute to the higher values found with that technique. We used in vitro binding to determine baseline levels of receptors and affinities for the other binding methods. In vitro binding is traditionally carried out on tissue homogenates that disrupt cell integrity and are thus rated lower on any scale of intactness. Furthermore, in this study and many others, to obtain valid numbers of B_{max}, the homogenate must be washed thoroughly to remove endogenous ligands and modulators of binding.

Neurochemical measures of transmitters or metabolites require highly disrupted homogenization. Often the substances to be measured must be extracted from the homogenate, eventually obtaining a purified substance far removed from the initial tissue. Nevertheless, transmitter and/or metabolite levels have the longest history of use as indicators of turnover or release, and in our hands, gave results equivalent to the methods using those tissue preparations that were substantially more intact (Table 3). In addition, the ligand used for the binding studies, raclopride, is commonly used for the assessment of dopamine D_2 binding sites, yet one cannot be certain that any one ligand or receptor subtype would be representative of transmitter release in general. However, the similarity of neurochemical and binding results seen here indicate that in these brain regions, ^{3}H-raclopride displacement might be a valid reflection of dopamine release. Andersen et al. (4) described a preparation that addresses transmitter release at prenatal ages. In that study, in vivo binding was assessed using ^{3}H-raclopride as an indirect measure of dopamine release (4).

More direct evidence of dopamine release was obtained in a separate study using in vivo microdialysis (3), again confirming that the in vivo binding technique using raclopride produces data analogous to direct assessment of dopamine.

Regarding the functions of the increased dopamine that is released in a novel environment, our observations are that isolated animals are initially more active. One reason for this is that when 10-day-old pups are in the nest (i.e., nonisolated), either with or without the mother, they huddle together and locomote very little. This correlation between activity and enhanced dopamine release has been well documented in a number of studies in adults (38,45) and in 10-day-old pups (26,27). The complete functional significance of the increased dopamine released during the 5-min isolation of the 10-day-old

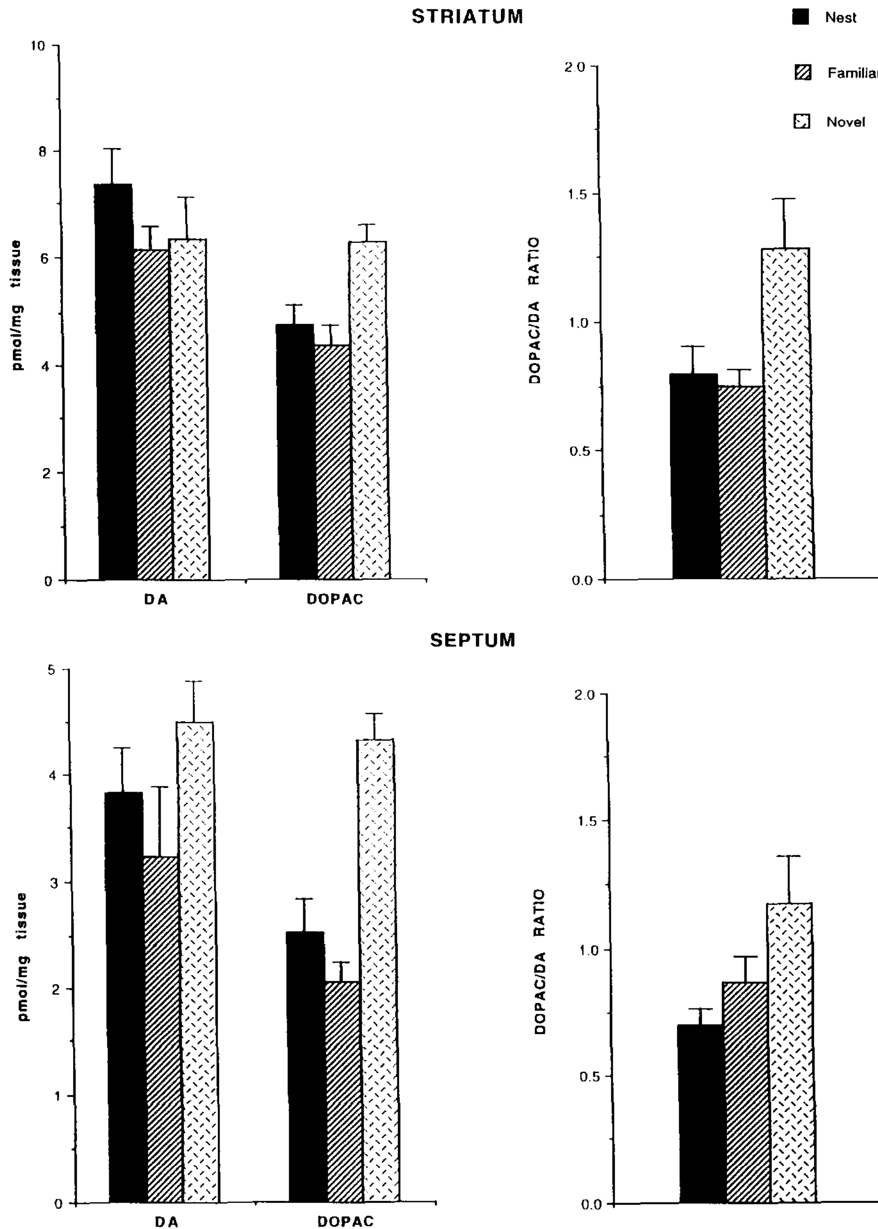

FIG. 4. (a) Mean striatal DA and DOPAC levels and DOPAC/DA ratio of pups taken from three conditions: directly from the nest, isolated for 5 min in a "familiar" condition, or isolated for 5 min in a "novel" condition. The values (in picomoles per milligram of tissue) represent the means (±SEM) of no less than eight subjects per group. Statistical analysis is discussed in the Results. (b) Mean septal DA and DOPAC levels and DOPAC/DA ratio of pups taken from three conditions: directly from the nest, isolated for 5 min in a "familiar" condition, or isolated for 5 min in a "novel" condition. The values (in picomoles per milligram of tissue) represent the means (±SEM) of no less than eight subjects per group. Statistical analysis is discussed in Results.

pups in the present study cannot be ascertained. However, future studies are planned in which levels of dopamine release will be assessed using in vivo microdialysis and correlated with observed behaviors during and following the isolation period.

In vivo microdialysis is increasingly becoming the method of choice in many investigations of transmitter release, including our own (23,27,28). However, in vivo microdialysis is limited to brain regions where transmitters are released in levels sufficient to be measured with current techniques. Further, these binding techniques can be used in brain regions that would be disrupted by microdialysis probe placement and can also be used to assess release in many sites of the same brain simultaneously. The in vivo and ex vivo binding techniques used in this study can be used to assess release of transmitters for which we lack sensitive assays, including many peptides or other transmitters that lack a strong focal innervation. For example, Insel (19) found decreased in vivo H3-RO-15-1788 binding to the GABA-benzodiazepine receptor in the neocortex, hippocampus, and inferior culliculi of rat pups after social isolation. In vitro binding of H3-RO-15-1788 in that same study demonstrated no change in B_{max} or K_d, indicating that the decreased in vivo binding did not reflect a change in receptor number or affinity. Insel's data support the hypothesis that the decrease in binding was due to decreased receptor availability. Likewise, in the current study, the decrease in in vivo and ex vivo binding, lack of change in in vitro binding, and increase in dopamine turnover all converged to indicate the activation of dopamine systems following isolation.

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