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Age-dependent effects of dopamine receptor inactivation on cocaine-induced behaviors in male rats: evidence of dorsal striatal D2 receptor supersensitivity

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Abstract

N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), which irreversibly inactivates dopamine (DA) receptors, causes pronounced age-dependent behavioral effects in rats. For example, EEDQ either augments or does not affect the DA agonist-induced locomotor activity of preweanling rats, while attenuating the locomotion of adolescent and adult rats. The twofold purpose of this study was to determine whether EEDQ would: (1) potentiate or attenuate the cocaine-induced locomotor activity of preweanling, adolescent, and adult rats, and (2) alter the sensitivity of surviving D2 receptors. Rats were treated with vehicle or EEDQ (2.5 or 7.5 mg/kg) on postnatal day (PD) 17, PD 39, and PD 84. In the behavioral experiments, saline- or cocaine-induced locomotion was assessed 24 h later. In the biochemical experiments, dorsal striatal samples were taken 24 h after vehicle or EEDQ treatment and later assayed for NPA-stimulated GTP γ S receptor binding, G protein-coupled receptor kinase 6 (GRK6), and β -arrestin-2 (ARRB2). GTP γ S binding is a direct measure of ligand-induced G protein activation, while GRK6 and ARRB2 modulate the internalization and desensitization of D2 receptors. Results showed that EEDQ potentiated the locomotor activity of preweanling rats, while attenuating the locomotion of older rats. NPA-stimulated GTP γ S binding was elevated in EEDQ-treated preweanling rats, relative to adults, indicating enhanced functional coupling between the G protein and receptor. EEDQ also reduced ARRB2 levels in all age groups, which is indicative of increased D2 receptor sensitivity. In sum, the present results support the hypothesis that D2 receptor supersensitivity is a critical factor mediating the locomotor potentiating effects of EEDQ in cocaine-treated preweanling rats.

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AUTHOR CONTRIBUTIONS

All authors approved the manuscript. *Conceptualization*, C.A.C. and S.A.M.; *Methodology*, S.A.M. and C.A.C.; *Investigation*, C.A.C., A.T., G.I.R., C.G.K., A.M.Y., S.E.E., and V.R.; *Formal Analysis*, S.A.M. and C.A.C.; *Writing – Original Draft*, S.A.M. and C.A.C.; *Writing – Review & Editing*, S.A.M., C.A.C., A.T., G.I.R., C.G.K., A.M.Y., S.E.E., and V.R.; *Visualization*, C.A.C. and S.A.M.; *Supervision*, S.A.M., and C.A.C.; *Funding Acquisition*, S.A.M. and C.A.C.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to report.

DATA ACCESSIBILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Graphical Abstract

Mean ($\pm$ SD) distance traveled scores converted to percent of vehicle controls at each age.

EEDQ augments the cocaine-induced locomotor activity of preweanling rats, while attenuating the locomotion of adolescent and adult rats. In the present study, we used NPA-stimulated GTP γ S receptor binding, and GRK6 and ARRB2 expression to test the hypothesis that D2 receptor supersensitivity is responsible for this age-dependent effect.

Keywords

N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ); ontogeny; GTP γ S receptor binding; G protein-coupled receptor kinase 6 (GRK6); β -arrestin-2 (ARRB2)

1 INTRODUCTION

Centrally acting drugs occasionally cause pronounced age-dependent behavioral effects in rats and mice (Spear, 1979, 2000). One of the most notable examples is the peptide-coupling

agent, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), which irreversibly inactivates dopamine (DA) D1 and D2 receptors, among other receptor types (Crawford, McDougall, Rowlett, & Bardo, 1992; Gnanalingham, Hunter, Jenner, & Marsden, 1994; Hamblin & Creese, 1983). Predictably, administering EEDQ 24 h before behavioral testing fully attenuates the D2 agonist-induced locomotor activity and stereotypic behaviors of adult rats. These psychopharmacological effects are apparent regardless of whether EEDQ is administered systemically (Arnt, Hyttel, & Meier, 1988; McDougall, Crawford, & Nonneman, 1992; Meller, Bordi, & Bohmaker, 1989) or is microinjected into the dorsal striatum (Bordi, Carr, & Meller, 1989; Cameron & Crocker, 1989; Der Ghazarian et al., 2012). Adolescent rats respond in the same manner as adults, since infusing EEDQ into the dorsal striatum on postnatal day (PD) 39 blocks the locomotor activating effects of D2 agonists (McDougall et al., 2014). In surprising contrast, systemic administration of EEDQ does not attenuate, and may augment, the D2 agonist-induced locomotor activity of preweanling rats (McDougall et al., 1992, 1993; Mestlin & McDougall, 1993). Indeed, microinjecting EEDQ into the dorsal striatum causes a significant potentiation of D2 agonist-induced locomotion in preweanling rats. This potentiation effect is restricted to drugs that directly [quinpirole or R-propyl-norapomorphine (NPA)] or indirectly (cocaine) stimulate D2 receptors, because EEDQ does not potentiate the locomotor activating effects of SKF82958 (a D1 agonist), MK-801 (an NMDA receptor channel blocker), or U50488 (a κ -opioid agonist) (Der Ghazarian et al., 2012, 2014).

In order to further determine receptor specificity, “protection” experiments are often used in conjunction with EEDQ. In these experiments, selective D1 (SCH23390) and/or D2 (e.g., sulpiride or raclopride) receptor antagonists are administered prior to EEDQ treatment, thus sparing specific receptor types from EEDQ-induced inactivation (Fuxe, Meller, Goldstein, Benfenati, & Agnati, 1986; Meller, Bohmaker, Goldstein, & Friedhoff, 1985). For example, various research groups have reported that the apomorphine- or NPA-induced stereotypic behaviors of adult rats are maintained if rats are pretreated with sulpiride or raclopride prior to EEDQ administration (Arnt et al., 1988; Cameron et al., 1989; Meller et al., 1989). DA antagonist pretreatment also protects the NPA-induced locomotor activity of adult rats from receptor inactivation (McDougall et al., 1992). Conversely, the ability of EEDQ to potentiate the NPA-induced locomotor activity of preweanling rats is only evident if D2 receptors are not protected from EEDQ (i.e., sulpiride or raclopride are not administered) (Der Ghazarian et al., 2014; McDougall et al., 1993). In other words, the dorsal striatal D2 receptors of preweanling rats must first be inactivated in order for D2 agonist-induced behavioral potentiation to occur on the test day (i.e., 24 h later).

The neural mechanisms responsible for this paradoxical potentiation effect are uncertain, but a likely possibility is that EEDQ causes D2 receptor supersensitivity in preweanling rats (for a mini-review, see Kostrzewa et al., 2018). If the surviving D2 receptors of EEDQ-treated preweanling rats are supersensitive, it would explain why NPA and other D2 agonists cause an enhanced behavioral response. EEDQ does not affect the DA receptor systems of preweanling and adult rats in an identical manner, as EEDQ causes a less profound reduction in the number of dorsal striatal D2 receptors of preweanling rats than adults (61% decline versus 80%, respectively; Crawford et al., 1992; see also Leff, Gariano, & Creese, 1984), and the rate of D2 receptor repopulation is greater in preweanling rats (Kula, George, &

Baldessarini, 1992; Leff et al., 1984). There is also evidence that the receptors remaining after EEDQ treatment are more likely to be in a high affinity state. Specifically, EEDQ increases the percentage of D2^{High} receptors in preweanling, adolescent, and adult rats, but the relative proportion of D2^{High} receptors is greater in EEDQ-treated preweanling rats than the other two age groups (McDougall et al., 2014). This finding is particularly relevant, because Seeman et al. (2005) have reported that a wide range of manipulations (pharmacological, physiological, and genetic) that result in D2 receptor supersensitivity also increase the proportion of D2^{High} receptors. Additional evidence supports the hypothesis that EEDQ causes D2 supersensitivity: (a) low-dose EEDQ treatment produces D1 receptor supersensitivity in adult rats (Trovero, Hervé, Blanc, Glowinski, & Tassin, 1992); (b) microinjecting EEDQ into the dorsal striatum of preweanling rats, but not adults, increases basal locomotor activity (Der Ghazarian et al., 2012, 2014); and (c) EEDQ reduces dorsal striatal DA levels (Crawford et al., 1992; Crawford, Rowlett, McDougall, & Bardo, 1994), an event that often leads to DA supersensitivity (Arnt & Hyttel, 1984; Carvalho et al., 2009; Farley, Baella, Wacan, Crawford, & McDougall, 2006). Therefore, there is converging evidence suggesting that EEDQ's ability to potentiate the D2 agonist-induced locomotor activity of preweanling rats is a product of D2 receptor supersensitivity.

A major goal of the present study was to determine the effects of EEDQ on neurochemical correlates known to be associated with D2 receptor supersensitivity. To assess functional D2 receptor supersensitivity (Seeman, Battaglia, Corti, Corsi, & Bruno, 2009), we examined whether EEDQ would increase NPA-stimulated GTP γ S binding. GTP γ S binding, which is a direct measure of ligand-induced G protein activation, is a reliable indicator of D2 receptor supersensitivity (Harrison & Traynor, 2003; Strange, 2010). We also measured G protein-coupled receptor kinase 6 (GRK6) levels and β -arrestin-2 (ARRB2) levels in the dorsal striatum, because treatment-induced reductions in GRK6 and ARRB2 are indicative of D2 receptor supersensitivity. The mechanism for this effect has been established, as activation of the D2 receptor causes GRK6 to phosphorylate the receptor, which, in turn, stimulates ARRB2 to bind with the G protein/receptor complex (Del'Guidice, Lemasson, & Beaulieu, 2011; Porter-Stransky & Weinshenker, 2017). ARRB2 binding causes internalization of the ligand-bound receptor, and a loss of receptor sensitivity, until the receptor is dephosphorylated (for reviews, see Gainetdinov, Premont, Bohn, Lefkowitz, & Caron, 2004; Kliewer, Reinscheid, & Schulz, 2017). A second goal of the present study was to determine whether a single pretreatment injection of EEDQ would potentiate the locomotor activity of cocaine-treated preweanling rats, while attenuating the locomotion of cocaine-treated adolescent and adult rats. For preweanling rats, it was hypothesized that EEDQ would: (a) potentiate cocaine-induced locomotor activity, (b) increase NPA-stimulated GTP γ S binding in the dorsal striatum, and (c) decrease dorsal striatal GRK6 and ARRB2 levels. This pattern of results would indicate that EEDQ increases the sensitivity of surviving dorsal striatal D2 receptors in preweanling rats. It was also hypothesized that EEDQ would attenuate the cocaine-induced locomotor activity of adolescent and adult rats, while having minimal effect on dorsal striatal GTP γ S binding as well as GRK6 and ARRB2 levels.

2 MATERIALS AND METHODS

2.1 Animals

Subjects were 344 male Sprague-Dawley rats. Rats tested on PD 80 ($N = 102$) were purchased from Charles River (Hollister, CA, USA). Rats tested on PD 18 ($N = 140$) or PD 40 ($N = 102$) were born and raised at California State University, San Bernardino (CSUSB). Litters were culled to 10 pups on PD 3 and weaned on PD 21. After weaning, rats were group housed with littermates. Food and water was freely available. The colony room was maintained at 22–24 °C and kept under a 12-h light/dark cycle. Subjects were cared for in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publications No. 80–23) under a research protocol approved by the Institutional Animal Care and Use Committee of CSUSB.

2.2 Apparatus

Behavioral testing was done in activity monitoring chambers that consisted of acrylic walls, a plastic floor, and an open top (Coulbourn Instruments, Whitehall, PA, USA). Each chamber included an X–Y photobeam array, with 16 photocells and detectors, that was used to determine distance traveled (a measure of locomotor activity). In order to equate for differences in body size (see also Campbell, Lytle, & Fibiger, 1969; Shalaby & Spear, 1980), PD 18 rats were tested in smaller chambers ($26 \times 26 \times 41$ cm) than the two older age groups ($41 \times 41 \times 41$ cm).

2.3 Drugs

(–)-Cocaine hydrochloride was dissolved in saline; whereas, EEDQ was dissolved in a 50% DMSO solution [1:1 (v/v) in distilled water]. NPA, which was used in the GTP γ S assays, was dissolved in distilled water containing 0.1% metabisulfite (an antioxidant). Drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA) and injected intraperitoneally (i.p.) at a volume of 2.5 ml/kg (PD 20) or 1 ml/kg (PD 40 and PD 85).

2.4 Behavioral Procedures

On the pretreatment day, which occurred on PD 17 ($N = 48$), PD 39 ($N = 48$), or PD 84 ($N = 48$), male rats were injected with vehicle or EEDQ (2.5 or 7.5 mg/kg, i.p.). On the test day, which occurred 24 h later (i.e., on PD 18, PD 40, or PD 85), rats ($n = 8$ rats per group) were injected with saline or cocaine (15 mg/kg, i.p.) immediately before being placed in activity chambers. In a separate experiment, preweanling rats ($N = 40$) were injected (i.p.) with vehicle or 7.5 mg/kg EEDQ on PD 17, and then injected 24 h later with saline or a low dose of cocaine (5 or 10 mg/kg, i.p.). There were a total of five groups: vehicle-saline, vehicle-cocaine (5 mg/kg), vehicle-cocaine (10 mg/kg), EEDQ-cocaine (5 mg/kg), and EEDQ-cocaine (10 mg/kg). After the second injection, rats ($n = 8$ rats per group) were immediately placed in activity chambers. In both experiments, distance traveled was measured for 120 min.

2.5 Homogenate [³⁵S]GTPγS binding assay

Experimentally naive male rats were injected with vehicle or EEDQ (2.5 or 7.5 mg/kg, i.p.) on PD 17 (*N* = 21), PD 39 (*N* = 21), or PD 84 (*N* = 21). After 24 h (i.e., on PD 18, PD 40, or PD 85), rats (*n* = 7 rats per group) were decapitated and the dorsal striatum (i.e., caudate-putamen) was dissected on dry ice and stored at −80 °C. On the day of assay, tissue was thawed on ice and samples were homogenized in 100 volumes of 50 mM Tris-HCl buffer (pH 7.4) for approximately 20 s using a Brinkman Polytron. Homogenates were centrifuged at 20,000 × *g* for 30 min. The pellet was resuspended in 100 volumes of the same buffer and centrifuged again at 20,000 × *g* for 30 min. The final pellet was suspended in approximately 20 volumes of buffer (pH 7.4) and incubated for 30 min at 30 °C to remove endogenous transmitter. Protein concentrations for the final pellet were determined using the Bio-Rad Protein Assay with BSA as the standard.

Agonist-effect curves of [³⁵S]GTPγS binding were performed in assay buffer (50 mM Tris-HCl, 120 mM NaCl) containing 30 μM GDP, 10–20 μg protein, and NPA (0.1 pM to 10 μM), or equivalent volumes of water. Non-specific binding was determined in the presence of 30 μM cold GTPγS. The tubes were pre-incubated for 15 min at 30 °C and then 0.1 nM [³⁵S]GTPγS was added. Following the addition of [³⁵S]GTPγS, tubes were incubated for an additional 30 min at 30 °C. The incubation period was ended by filtering the contents of the tubes using glass fiber filters. Net NPA-stimulated [³⁵S]GTPγS binding values were calculated by subtracting basal binding values (without agonist) from NPA-stimulated values and dividing by basal values. Agonist potency (pEC₅₀) and agonist efficacy (E_{max}) were determined by iterative nonlinear regression fitting using Prism (Graph Pad Software).

2.6 β-Arrestin and GRK6 assays

On PD 17 (*N* = 31), PD 39 (*N* = 33), or PD 84 (*N* = 33), male rats were injected with vehicle or EEDQ (2.5 or 7.5 mg/kg). After 24 h, rats (*n* = 10–12 per group) were killed by rapid decapitation and dorsal striatal sections were dissected and stored as described above. Frozen striatal sections were sonicated in 20 nM Tris buffer containing 1% NP40 and a protease inhibitor cocktail. After sonication, samples were centrifuged at 10,000 × *g* for 15 min at 4 °C. Protein concentrations for the final pellet were determined using the Bio-Rad Protein Assay with BSA as the standard. ARRB2 and GRK6 levels were assessed using commercially available enzyme-linked immunosorbent assay (ELISA) kits (CBS-EL002135RA and CBS-EL009927RA) according to the manufacturer's instructions (Cusabio Biotech, Houston, TX, USA). Optical density values were made (absorbance readings at 450 nm, with a wavelength correction reading at 540 nm) using a micro plate reader (Multiskan Sky, Thermo Scientific, Waltham, MA, USA).

2.7 Data analysis

In order to minimize litter effects, no more than one subject per litter was assigned to a given group (Holson & Pearce, 1992). For the first behavioral experiment, distance traveled data were analyzed using a 3 × 3 × 2 × 12 (age × pretreatment × drug × time block) repeated measures analysis of variance (ANOVA); whereas, a 5 × 12 (group × time block) repeated measures ANOVA was used in the second behavioral experiment. When the assumption of sphericity was violated, as determined by Mauchly's test of sphericity, the Huynh-Feldt

epsilon statistic was used to adjust degrees of freedom (Huynh & Feldt, 1976). Corrected degrees of freedom were rounded to the nearest whole number and are indicated by a superscripted “a” in the parenthetical statistical reports. When further analyzing statistically significant higher order interactions, the mean square error terms (i.e., MS_{error}) used for Tukey calculations were based on separate one- or two-way ANOVAs at each time block. For the GTP γ S binding experiments a logarithmic transformation of the E_{max} and pEC_{50} data was conducted ($Y' = \log_{10} Y$). E_{max} and pEC_{50} data from the GTP γ S binding assays, as well as the untransformed data from the ARRB2 and GRK6 assays (pg/mg protein), were analyzed using a 3×3 (age $\times$ pretreatment) between-subjects ANOVAs. Tukey tests were also used for post hoc comparisons involving the biochemistry data. Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 24.0 (IBM, Armonk, NY, USA). Main effects, interactions, and post hoc tests were considered significant at $P < 0.05$. In all cases, rats were randomly assigned to groups and experimenters were blind to drug treatment conditions.

3 RESULTS

3.1 Ontogenetic differences in locomotor activity: untransformed data

Overall, an omnibus ANOVA showed that age interacted with the pretreatment, drug, and time variables to affect distance traveled scores [Age $\times$ Pretreatment $\times$ Drug interaction, $F_{4,126} = 15.24$; $N = 144$; $P = 0.001$; a Age $\times$ Pretreatment $\times$ Drug $\times$ Time Block interaction, $F_{19,610} = 1.60$; $N = 144$; $P = 0.049$]. To further break apart statistically significant three- and four-way interactions, separate lower order ANOVAs were conducted at each age.

3.1.1 Preweanling rats (PD 18)—Among preweanling rats (upper graphs, Figure 1), cocaine significantly enhanced locomotor activity [Drug main effect, $F_{1,42} = 48.80$; $N = 48$; $P = 0.001$], an effect that was apparent in both EEDQ pretreatment conditions [Pretreatment $\times$ Drug interaction, $F_{2,42} = 7.95$; $N = 48$; $P = 0.001$; and Tukey tests, $P = 0.011$ and $P = 0.001$]. In saline-treated preweanling rats (upper left graph, Figure 1), EEDQ did not alter distance traveled scores; however, 7.5 mg/kg EEDQ potentiated the locomotor activity of rats treated with 15 mg/kg cocaine on time blocks 5–12 (upper right graph, Figure 1) [a Pretreatment $\times$ Drug $\times$ Time block interaction, $F_{13,265} = 1.91$; $N = 48$; $P = 0.031$; and Tukey tests, $P = 0.012$ to $P = 0.001$].

3.1.2 Adolescent rats (PD 40)—Overall, 2.5 and 7.5 mg/kg EEDQ significantly reduced the locomotor activity of adolescent rats [Pretreatment main effect, $F_{2,42} = 29.40$; $N = 48$; $P = 0.001$; and Tukey tests, $P = 0.001$]. Cocaine increased the distance traveled scores of adolescent rats, relative to their saline-treated controls [Drug main effect, $F_{1,42} = 57.60$; $N = 48$; $P = 0.001$], but this effect was only apparent in rats pretreated with vehicle (compare the two middle graphs, Figure 1) [Pretreatment $\times$ Drug interaction, $F_{2,42} = 5.90$; $N = 48$; $P = 0.006$; and Tukey test, $P = 0.001$]. Separate analyses of saline-treated adolescent rats indicated that 2.5 mg/kg EEDQ depressed locomotion on time blocks 1, 2, 4, and 11; whereas, 7.5 mg/kg EEDQ reduced locomotion on time blocks 1–6 and time blocks 8 and 10 (middle left graph, Figure 1) [a Pretreatment $\times$ Time block interaction, $F_{19,204} = 4.51$; $N = 24$; $P = 0.001$; and Tukey tests, $P = 0.048$ to $P = 0.001$]. As expected, both doses of EEDQ

significantly reduced the locomotor activity of cocaine-treated adolescent rats on all 12 time blocks (middle right graph, Figure 1) [^aPretreatment $\times$ Time block interaction, $F_{5,53} = 3.31$; $N = 24$; $P = 0.011$; and Tukey tests, $P = 0.044$ to $P = 0.001$].

3.1.3 Adult rats (PD 85)—Adult rats showed the same general pattern of behavioral effects as adolescent rats, since cocaine only increased the locomotor activity of adult rats pretreated with vehicle (compare the two bottom graphs, Figure 1) [Pretreatment $\times$ Drug interaction, $F_{2,42} = 28.06$; $N = 48$; $P = 0.001$; and Tukey test, $P = 0.001$]. More specifically, both doses of EEDQ significantly reduced the distance traveled scores of cocaine-treated adult rats (time blocks 1–12) and saline-treated adult rats (time blocks 1–3 and time block 7) [^aPretreatment $\times$ Drug $\times$ Time block interaction, $F_{8,158} = 3.67$; $N = 48$; $P = 0.001$; and Tukey tests, $P = 0.021$ to $P = 0.001$].

3.1.4 Inter-age comparisons—In terms of untransformed distance traveled data, preweanling rats in the vehicle-saline control group exhibited less locomotor activity than similarly treated adolescent (time block 1) and adult rats (time blocks 1–3 and time block 7) (compare left graphs, Figure 1) [Age $\times$ Pretreatment interaction, $F_{4,63} = 5.20$; $N = 72$; $P = 0.001$; ^aAge $\times$ Pretreatment $\times$ Time block interaction, $F_{28,438} = 2.22$; $N = 72$; $P = 0.001$; and Tukey tests, $P = 0.033$ to $P = 0.001$]. No age differences were apparent when EEDQ-pretreated rats were given saline. When injected with cocaine, vehicle-pretreated adolescent and adult rats had greater distance traveled scores than preweanling rats on time blocks 2–12 (compare right graphs, Figure 1) [Age $\times$ Pretreatment interaction, $F_{4,63} = 32.82$; $N = 72$; $P = 0.001$; ^aAge $\times$ Pretreatment $\times$ Time block interaction, $F_{17,261} = 2.20$; $N = 72$; $P = 0.005$; and Tukey tests, $P = 0.008$ to $P = 0.001$]. Conversely, cocaine-treated preweanling rats injected with 7.5 mg/kg EEDQ had greater distance traveled scores than similarly treated adult rats on time blocks 1–12 and similarly treated adolescent rats on time blocks 6–12 [Tukey tests, $P = 0.028$ to $P = 0.001$].

3.2 Ontogenetic differences in locomotor activity: percent of same-age vehicle controls

The effects of EEDQ on both saline- and cocaine-induced locomotor activity are more readily apparent if data are transformed to percent of vehicle controls (i.e., % of the vehicle-saline and vehicle-cocaine groups) at each age (Figure 2). An omnibus ANOVA indicated that the age variable interacted with pretreatment condition and drug to affect performance [Age $\times$ Pretreatment $\times$ Drug interaction, $F_{4,126} = 3.77$; $N = 144$; $P = 0.006$]. A separate two-way ANOVA comparing only saline-treated rats (left box plots, Figure 2) showed that the %vehicle control distance traveled (%VcDT) data of preweanling rats did not vary according to pretreatment condition. In contrast, saline-treated adolescent and adult rats exhibited a significant reduction in %VcDT when pretreated with 2.5 or 7.5 mg/kg EEDQ [Age $\times$ Pretreatment interaction, $F_{4,63} = 3.43$; $N = 72$; $P = 0.013$; and Tukey tests, $P = 0.012$ to $P = 0.001$]. Among cocaine-treated preweanling rats (upper right box plots, Figure 2), 7.5 mg/kg EEDQ significantly potentiated %VcDT when compared to preweanling rats injected with either vehicle or 2.5 mg/kg EEDQ [Age $\times$ Pretreatment interaction, $F_{4,63} = 20.54$; $N = 72$; $P = 0.001$; and Tukey tests, $P = 0.014$ to $P = 0.001$]. Nearly opposite effects occurred in adolescent and adult rats (middle and lower graphs, Figure 2), as both doses of EEDQ (2.5

and 7.5 mg/kg) significantly attenuated the %VcDT scores of cocaine-treated rats [Tukey tests, $P = 0.002$ to $P = 0.001$].

3.3 Effects of EEDQ on low-dose cocaine-induced locomotor activity in preweanling rats

3.3.1 Untransformed data—Vehicle-pretreated preweanling rats injected with cocaine (5 or 10 mg/kg) exhibited greater distance traveled scores than vehicle controls (i.e., the vehicle-saline group) over the first 30 min of the testing session (Figure 3) [Group $\times$ Time block interaction, $F_{9,165} = 7.60$; $N = 40$; $P = 0.001$; and Tukey tests, $P = 0.026$ to $P = 0.001$]. Overall, EEDQ potentiated cocaine's actions, as rats treated with both EEDQ and cocaine (5 or 10 mg/kg) had greater distance traveled scores than rats treated with cocaine alone on time blocks 4–12 [Tukey tests, $P = 0.016$ to $P = 0.001$].

3.3.2 Percent of vehicle controls—When data are transformed to percent of vehicle controls (%VcDT), preweanling rats treated with both EEDQ and cocaine (5 or 10 mg/kg) exhibited 6- to 7-fold more locomotor activity than rats in the vehicle-saline group (Figure 4). When compared to preweanling rats given cocaine alone (i.e., the vehicle-cocaine groups), EEDQ significantly potentiated the distance traveled scores of cocaine-treated rats [Group main effect, $F_{4,35} = 17.80$; $N = 40$; $P = 0.001$; and Tukey tests, $P = 0.003$ and $P = 0.001$].

3.4 NPA-stimulated [35 S]GTP γ S binding in the dorsal striatum

The efficacy (i.e., $E_{\max}$) of NPA-stimulated [35 S]GTP γ S specific binding in the dorsal striatum was significantly greater in preweanling rats than adults (Figure 5) [Age main effect, $F_{2,54} = 3.98$; $N = 63$; $P = 0.025$; and Tukey test, $P = 0.02$]. Treating rats with 2.5 mg/kg EEDQ ($x = 30.38$, $SD = 13.1$) or 7.5 mg/kg EEDQ ($x = 30.90$, $SD = 13.1$) increased dorsal striatal $E_{\max}$ values relative to vehicle controls ($x = 15.23$, $SD = 5.7$) [Drug main effect, $F_{2,54} = 15.54$; $N = 63$; $P = 0.001$; and Tukey tests, $P = 0.001$], although the two EEDQ conditions did not differ from each other. When data from the EEDQ conditions were combined, it was apparent that EEDQ increased the efficacy of NPA-stimulated [35 S]GTP γ S specific binding of preweanling rats when compared to either vehicle-treated preweanling rats or EEDQ-treated adult rats [Age $\times$ Pretreatment interaction, $F_{2,57} = 3.81$; $N = 63$; $P = 0.028$; and Tukey tests, $P = 0.004$ and $P = 0.001$]. Potency (i.e., pEC_{50}) of NPA-stimulated [35 S]GTP γ S specific binding did not vary according to age or pretreatment condition (Table 1).

3.5 β -Arrestin (ARRB2) levels in the dorsal striatum

When compared to the vehicle controls ($x = 925.0$, $SD = 342$), rats pretreated with 2.5 mg/kg EEDQ ($x = 778.4$, $SD = 269$) or 7.5 mg/kg EEDQ ($x = 754.8$, $SD = 231$) had significantly reduced ARRB2 levels in the dorsal striatum (Figure 6) [Pretreatment main effect, $F_{2,86} = 5.92$; $N = 95$; $P = 0.004$; and Tukey tests, $P = 0.004$ and $P = 0.001$]. ARRB2 levels increased progressively according to age, as adult rats had significantly greater values than preweanling rats, while adolescent rats were intermediate between and significantly different from the other two age groups [Age main effect, $F_{2,86} = 75.28$; $N = 95$; $P = 0.001$; and Tukey tests, $P = 0.001$]. The Age $\times$ Pretreatment interaction was not statistically significant [$P = 0.21$].

3.6 GRK6 levels in the dorsal striatum

EEDQ did not affect dorsal striatal GRK6 content, although adult rats had significantly greater GRK6 levels than either of the younger age groups (Figure 7) [Age main effect, $F_{2,86} = 72.25$; $N = 95$; $P = 0.001$; and Tukey tests, $P = 0.023$ and $P = 0.001$]. GRK6 levels were also elevated in adolescent rats relative to preweanling rats.

4 DISCUSSION

When administered intracerebrally or systemically, EEDQ causes a substantial reduction of D1 and D2 receptors in the dorsal striatum. For example, we previously reported that an i.p. injection of 7.5 mg/kg EEDQ reduces the number of D1 and D2 binding sites in adult and preweanling rats by 82–86% (A) and 61–69% (P), respectively (Crawford et al., 1992). In the present study, this magnitude of receptor inactivation was sufficient to significantly attenuate both the basal and cocaine-induced locomotor activity of adult rats (see Figures 1 and 2). Similar effects have been reported before using other DA agonists, as EEDQ blocks the apomorphine-, NPA-, and quinpirole-induced stereotypy and locomotor activity of adult rats (Arnt et al., 1988; Bordi et al., 1989; Cameron & Crocker, 1989; Der Ghazarian et al., 2012; McDougall et al., 1992, 2014; Meller et al., 1989). In clear contrast, EEDQ did not block, but instead potentiated, the cocaine-induced locomotor activity of preweanling rats. This potentiation effect was evident in two separate experiments and across a range of cocaine doses (5, 10, and 15 mg/kg). A similar potentiation effect was observed when cocaine was microinjected into the dorsal striatum of preweanling rats (Der Ghazarian et al., 2014). Although past studies typically report that systemically administered EEDQ neither potentiates nor attenuates DA agonist-induced locomotor activity (McDougall et al., 1992, 1993; Mestlin & McDougall, 1993), the present study provides the first unambiguous evidence, using systemic injections, that EEDQ augments DA agonist-induced locomotion in preweanling rats. This phenomenon is unique to early ontogeny, because EEDQ affects adolescent rats in the same manner as adults (see also McDougall et al., 2014).

A disadvantage of using EEDQ as a pharmacological tool is the lack of receptor specificity. For example, EEDQ inactivates D1 and D2 receptors, as well as 5-HT_{1A} and 5-HT_{2A}, α_2 -adrenergic, and GABA_A receptors (Kettle, Cheetham, Martin, Prow, & Heal, 1999; Miller, Lumpkin, Galpern, Greenblatt, & Shader, 1991; Ribas, Miralles, Escribá, & García-Sevilla, 1998; Vinod, Subhash, & Srinivas, 2001). Two strategies are commonly employed to mitigate the lack of specificity: (1) rats can be tested using a variety of dopaminergic and nondopaminergic compounds, and (2) the receptor(s) of interest can be protected from EEDQ-induced inactivation by pretreating rats with selective reversible antagonists (Fuxe et al., 1986; Meller et al., 1985). Using both of these strategies, we have shown that the D2 receptor is critical for the potentiation effect exhibited by preweanling rats. Specifically, locomotor potentiation is only evident when drugs that directly or indirectly stimulate D2 receptors are used (i.e., no potentiation is evident when D1, NMDA, or κ -opioid compounds are administered; Der Ghazarian et al., 2012, 2014; McDougall et al., 1993). Moreover, DA agonist-induced potentiation only occurs in preweanling rats if D2 receptors are unprotected from EEDQ-induced inactivation (Der Ghazarian et al., 2014; McDougall et al., 1993).

The purpose of the present study was not to revisit the issue of EEDQ and receptor specificity. That being said, cocaine has a high affinity for both the DA and serotonin (5-HT) transporters (Howell & Kimmel, 2008), and both of these neurotransmitter systems modulate motoric responding (Geyer, 1996; Hauber, 1998; Tzschentke, 2001). Because EEDQ binds to various 5-HT receptor subtypes (Kettle et al., 1999; Vinod et al., 2001), it is possible that 5-HT receptor inactivation contributed to the locomotor potentiation exhibited by preweanling rats. In this regard, it is noteworthy that D-amphetamine, which has little impact on the 5-HT transporter, does not cause a potentiated locomotor response in EEDQ-treated preweanling rats (Crawford, McDougall, & Bardo, 1994). Rather than being due to 5-HT involvement, however, it is likely that the different behavioral actions of D-amphetamine and cocaine are a consequence of the DA pools that each drug relies on. Specifically, cocaine acts as a transport inhibitor, while D-amphetamine enhances DA release through actions involving both the plasma membrane DA transporter and the vesicular monoamine transporter type 2 (VMAT₂) (Fleckenstein & Hanson, 2003; McMillen, 1983). Thus, cocaine increases extracellular DA via vesicular stores, whereas D-amphetamine primarily relies on cytosolic pools (Kuczenski, 1983). Among its other actions, EEDQ dramatically reduces the amount of DA in cytosolic pools (Crawford et al., 1992; Crawford, Rowlett et al., 1994), thus potentially explaining the behavioral dichotomy between D-amphetamine and cocaine in EEDQ-treated preweanling rats.

Although it has been established that D2 receptors are primarily responsible for EEDQ's paradoxical potentiation effect, the neural mechanisms underlying this effect have not been fully elucidated. One possibility is that preweanling rats have a large D2 receptor reserve that is sufficient to compensate for the receptors inactivated by EEDQ. This explanation is unlikely, however, because: (a) adult rats do not have a D2 receptor reserve (Arnt et al., 1988; Meller, Enz, & Goldstein, 1988), (b) D2 receptor densities in the dorsal striatum are similar during the late preweanling period and adulthood (Murrin & Zeng, 1986; Rao, Molinoff, & Joyce, 1991; Tarazi & Baldessarini, 2000), thus making a D2 receptor reserve unlikely in the younger age group, and (c) a hypothetical D2 receptor reserve is insufficient to explain the *potentiation* of behavioral responding. Instead, we have hypothesized that the D2 receptors remaining after EEDQ treatment are in a supersensitive state and, as a consequence, are capable of mediating a potentiated locomotor response. The presumed reason why EEDQ only potentiates the locomotor activity of preweanling rats and not adults is twofold: (1) fewer D2 receptors are inactivated by EEDQ in preweanling rats when compared to adults (Crawford et al., 1992; Leff et al., 1984), and (2) among the surviving dorsal striatal D2 receptors, preweanling rats have a greater percentage that are in a high affinity state (McDougall et al., 2014).

To further examine this supersensitivity hypothesis, GTP γ S binding, as well as GRK6 and ARRB2 levels, were measured in EEDQ- and vehicle-treated preweanling, adolescent, and adult rats. Agonist-stimulated GTP γ S binding is positively related to the amount of dorsal striatal D2^{High} receptors (Seeman et al., 2009), and is indicative of D2 receptors in a supersensitive state (Harrison & Traynor, 2003; Strange, 2010). In the present study, EEDQ increased NPA-stimulated GTP γ S binding, which is consistent with past studies showing elevated GTP γ S binding after chronic haloperidol treatment and 6-hydroxydopamine lesions (Geurts, Hermans, Cumps, & Maloteaux, 1999; Geurts, Hermans, & Maloteaux, 1999).

Importantly, the EEDQ-induced increase in NPA-stimulated GTP γ S binding was most pronounced in preweanling and adolescent rats (a 295% and 174% increase, respectively), as EEDQ only caused a nonsignificant increase in the GTP γ S binding of adult rats (151%). Reductions in ARRB2 are also associated with D2 receptor supersensitivity, as manipulations that reduce dorsal striatal and accumbal ARRB2 levels result in an enhanced behavioral response to acute cocaine or methamphetamine treatment (Gaval-Cruz et al., 2016; Oda et al., 2015). In the present study, EEDQ decreased ARRB2 levels, but this effect did not vary according to age. Interestingly, EEDQ did not alter GRK6 levels, despite reports that: (a) chronic haloperidol treatment decreases GRK6 levels in monkeys (Hernandez et al., 2019) and (b) GRK6-deficient mice show an enhanced behavioral response to amphetamine and cocaine (Gainetdinov et al., 2003). Nonetheless, the same pattern of results described here (i.e., decreased ARRB2 levels and unchanged GRK6 levels) were reported by Oda and colleagues (2015), who found that repeated haloperidol treatment increased the sensitivity of D2 receptors. Thus, it appears that reductions in ARRB2 alone are reflective of D2 receptor supersensitivity (Oda et al., 2015).

In a broader developmental context, EEDQ is one of many compounds, from multiple drug classes (e.g., DA agonists, κ -opioid agonists, NMDA receptor channel blockers, etc.), that produce dramatically different behavioral effects across ontogeny (Charntikov, Halladay, Herbert, Marquez, & McDougall, 2008; McDougall, Moran, Baum, Apodaca, & Real, 2017; Shalaby & Spear, 1980). Although each situation is unique, it is likely that these age-dependent psychopharmacological effects result from maturational changes in the neural mechanisms underlying drug action and behavior (for reviews, see Andersen, 2005; Spear, 1979, 2000). In terms of the DA system, many elements (e.g., DA content, VMAT₂, and plasma membrane DA transporters) exhibit a linear increase from the neonatal period into adulthood (Broaddus & Bennett, 1990; Giorgi et al., 1987; Kuperstein, Eilam, & Yavin, 2008; Truong et al., 2005). Consistent with this pattern of development, the dorsal striatal ARRB2 and GRK6 protein levels of nondrug-treated rats increased in a step-wise and progressive manner from the preweanling period, through adolescence, and into adulthood (Figures 6 and 7; see also Gurevich, Benovic, & Gurevich, 2002). DA receptors show a different maturational pattern, as dorsal striatal D1 and D2 receptors increase in number from birth until the end of the preweanling period (PD 21), when adult-like levels are reached (Murrin & Zeng, 1986; Rao et al., 1991; Zeng, Hyttel, & Murrin, 1988). Interestingly, however, there is a transient overproduction of D1 and D2 receptors during adolescence (for reviews, see Andersen, 2003; Tarazi & Baldessarini, 2000), which may have clinical significance (Andersen & Teicher, 2000). A similar developmental pattern was not evident when D2 receptor functioning was assessed, since the NPA-stimulated GTP γ S receptor binding of vehicle-treated preweanling, adolescent, and adult rats did not vary according to age (Figure 5).

In terms of constraints, interpretation of the present results is somewhat limited because sex was not included as a variable in the study. Male and female preweanling rats do not typically respond differently to DA-acting drugs, including cocaine (Kozanian, Gutierrez, Mohd-Yusof, & McDougall, 2012; McDougall, Eaton, Mohd-Yusof, & Crawford, 2015; Snyder, Katovic, & Spear, 1998); however, adult rats often exhibit cocaine-induced sex differences (e.g., Festa et al., 2004; McDougall et al., 2015; Schindler & Carmona, 2002).

Therefore, future research should examine how sex and age interact to affect the DA agonist-induced responding of EEDQ-treated rats.

In conclusion, EEDQ pretreatment potentiated the cocaine-induced locomotor activity of preweanling rats, while attenuating the cocaine-induced locomotion of adolescent and adult rats. We had originally hypothesized that this potentiation effect, observed only in preweanling rats, was due to DA receptor supersensitivity. Consistent with this idea, the surviving D2 receptors of preweanling rats, when compared to adults, are more likely to be in a high affinity state (McDougall et al., 2014), and the dorsal striatal DA receptors of preweanling rats exhibit enhanced functional coupling (i.e., increased GTP γ S binding). The EEDQ-induced reduction in dorsal striatal ARRB2 is also suggestive of enhanced D2 receptor sensitivity, but this effect was evident in all age groups. Therefore, it is likely that the differential rate of receptor depletion (i.e., EEDQ inactivates a greater percentage of D2 receptors in adult rats) plays an important role in the EEDQ-induced augmentation of D2 receptor-mediated behaviors. Taken together, the present results, as well as our previously reported findings on D2^{High} receptors, support the hypothesis that D2 receptor supersensitivity is one of the critical factors responsible for EEDQ's ability to potentiate the cocaine-induced locomotor activity of preweanling rats. In terms of translational relevance, age-dependent differences in D2 receptor supersensitivity may be an important factor influencing the onset and maintenance of various human clinical states, including schizophrenia, attention-deficit hyperactivity disorder, Tourette's syndrome, and substance abuse (for a mini-review, see Kostrzewa et al., 2018). The present results suggest that D2 receptor supersensitivity may be more pronounced during early development and cause qualitatively different behavioral effects across ontogeny.

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Significance Statement

Young and old animals typically respond to drugs in a similar manner; however, there are rare situations when drugs induce different behavioral effects depending on the developmental stage of the organism. One such example is EEDQ, which irreversibly binds to dopamine D2 receptors. In adult rats, EEDQ blocks the actions of stimulant drugs, but in infant rats EEDQ causes these drugs to have exaggerated effects. Using behavioral and biochemical assays, we examined whether age-dependent changes in D2 receptor sensitivity may be responsible for these behavioral effects. Our results support the hypothesis that EEDQ causes D2 receptor supersensitivity in young rats.

FIGURE 1.

Mean ($\pm$ SD) distance traveled scores of preweanling, adolescent, and adult rats ($n = 8$ rats per group) injected with saline or cocaine (15 mg/kg, i.p.) immediately before testing. On the pretreatment day, which occurred 24 h earlier, rats had been injected with vehicle or EEDQ (2.5 or 7.5 mg/kg, i.p.). 'a' Significant difference between same-age rats receiving vehicle and 7.5 mg/kg EEDQ. 'b' Significant difference between same-age rats receiving vehicle and 2.5 mg/kg EEDQ. 'c' Significant difference between older (adolescents and adults) and preweanling rats in the control groups (i.e., Vehicle-Saline or Vehicle-Cocaine groups). 'd' Significant difference between preweanling and adolescent rats in the 7.5 mg/kg EEDQ-Cocaine group. 'e' Significant difference between preweanling and adult rats in the 7.5 mg/kg EEDQ-Cocaine group.

FIGURE 2.

Mean ($\pm$ SD) distance traveled scores converted to percent of vehicle controls at each age (these are the same rats as described in Figure 1). 'a' Significantly different from same-age rats in their vehicle control group (represented by the dashed line). 'b' Significantly different from same-age rats in their vehicle control group (represented by the dashed line). 'c' Significantly different from same-age rats in the 2.5 mg/kg EEDQ-Vehicle group.

FIGURE 3.

Mean ($\pm$ SD) distance traveled scores of preweanling rats ($n = 8$ rats per group) injected with saline or cocaine (5 or 10 mg/kg, i.p.) immediately before testing. On the pretreatment day, which occurred 24 h earlier, rats had been injected with vehicle or EEDQ (7.5 mg/kg, i.p.). 'a' Significant difference between the Vehicle-Saline group and rats receiving cocaine alone (i.e., the Vehicle-5 mg/kg Cocaine and Vehicle-10 mg/kg Cocaine groups). 'b' Significant difference between the vehicle- and EEDQ-treated rats tested with 5 mg/kg cocaine. 'c' Significant difference between the vehicle- and EEDQ-treated rats tested with 10 mg/kg cocaine.

FIGURE 4.

Mean ($\pm$ SD) distance traveled scores of preweanling rats converted to percent of vehicle controls (these are the same rats as described in Figure 3). 'a' Significantly different from the Vehicle-Saline group (represented by the dashed line). 'b' Significantly different from the Vehicle-Cocaine (5 mg/kg) group. 'c' Significantly different from the Vehicle-Cocaine (10 mg/kg) group.

FIGURE 5.

Mean efficacy ($E_{\max}$) of NPA-stimulated [^{35}S]GTP γ S specific binding in the dorsal striatum of preweanling, adolescent, and adult rats ($n = 7$ per group). On the pretreatment day, which occurred 24 h earlier, rats had been injected with vehicle or EEDQ (2.5 or 7.5 mg/kg, i.p.). $E_{\max}$ is expressed as the percentage of stimulation above basal binding. “V+E+E” refers to mean $E_{\max}$ values collapsed across the vehicle and two EEDQ groups. ‘a’ Significantly different from adult rats. ‘b’ Significantly different from vehicle-treated preweanling rats. ‘c’ Significantly different from EEDQ-treated adult rats.

FIGURE 6.

Mean β -arrestin levels (pg/mg protein) in the dorsal striatum of preweanling, adolescent, and adult rats ($n = 10$ – 12 per group). On the pretreatment day, which occurred 24 h earlier, rats had been injected with vehicle or EEDQ (2.5 or 7.5 mg/kg, i.p.). “V+E+E” refers to mean β -arrestin values collapsed across the vehicle and two EEDQ groups. ‘a’ Significantly different from adult rats. ‘b’ Significantly different from adolescent rats.

FIGURE 7.

Mean GRK6 levels (pg/mg protein) in the dorsal striatum of preweanling, adolescent, and adult rats ($n = 10\text{--}12$ per group). On the pretreatment day, which occurred 24 h earlier, rats had been injected with vehicle or EEDQ (2.5 or 7.5 mg/kg, i.p.). “V+E+E” refers to mean GRK6 values collapsed across the vehicle and two EEDQ groups. ‘a’ Significantly different from adult rats. ‘b’ Significantly different from adolescent rats.

Table 1

Mean (SD) potency (pEC₅₀) of NPA-stimulated [³⁵S]GTPγS specific binding in the dorsal striatum of EEDQ-treated preweanling, adolescent, and adult rats (*n* = 7 per group)

Pretreatment	Age			
	Preweanling	Adolescent	Adult	x (P+A+A)
Vehicle	6.93 (0.88)	6.63 (1.12)	6.74 (0.35)	6.74 (0.82)
2.5 mg/kg EEDQ	6.54 (1.34)	6.01 (0.78)	6.46 (0.95)	6.37 (1.03)
7.5 mg/kg EEDQ	6.74 (0.77)	6.46 (1.37)	6.05 (1.24)	6.35 (1.18)
x (V+E+E)	6.53 (1.04)	6.51 (1.13)	6.42 (0.92)	

Note: x (V+E+E) refers to mean E_{max} values collapsed across the vehicle and two EEDQ groups; x (P+A+A) refers to mean E_{max} values collapsed across the preweanling, adolescent, and adult age groups.