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AGE-DEPENDENT EFFECTS OF EEDQ ON COCAINE-INDUCED LOCOMOTOR ACTIVITY AND D2 RECEPTOR SUPERSENSITIVITY

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A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

In Partial Fulfillment

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in

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ABSTRACT

The neurochemical changes occurring between the preweanling period and adolescence could be crucial for understanding the role development plays in the manifestation of psychotic behaviors. N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) fully attenuates the DA agonist-induced behaviors of adult rats, while potentiating the DA agonist-induced locomotor activity of preweanling rats. My specific hypotheses were as follows: (1) Systemically administered EEDQ would block the cocaine-induced locomotor activity of adult rats. (2) Systemically administered EEDQ would potentiate the cocaine-induced locomotion of preweanling rats. (3) EEDQ would increase the E_{max} values (a measure of D2 receptor sensitivity) of preweanling rats, but not adolescent or adult rats. And, (4) EEDQ would reduce dorsal striatal β-arrestin-2 (ARRB2) and GRK6 levels (measures of D2 receptor sensitivity) of preweanling rats. Behavioral results were as expected, because EEDQ attenuated the locomotion of adult and adolescent rats, while EEDQ potentiated locomotor activity of preweanling rats. EEDQ enhanced the GTPγS binding of preweanling rats, while depressing ARRB2 levels. These results are consistent with the overarching hypothesis that EEDQ causes DA supersensitivity in preweanling rats. Thus, it is here proposed that EEDQ inactivates a significant number of D2 receptors in preweanling rats, but that the remaining D2 receptors are supersensitive and capable of mediating a potentiated locomotor response.

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CHAPTER ONE

INTRODUCTION

The dopamine (DA) system primarily mediates motor movement, cognition, and emotional behavior (Keeler, Pretsell, & Robbins, 2014). Activation of the DA system causes distinct actions depending on which receptors are targeted. For example, under- or over-stimulation of D1 receptors results in mood and cognitive impairments (Avery & Krichmar, 2015); whereas, overproduction of D2 receptors in the nucleus accumbens (NAc) may cause psychotic behaviors to emerge (Yoon, Minzenberg, Raouf, D'Esposito, & Carter, 2013). D3 receptors, which are preferentially found in the hypothalamus, limbic areas, substantia nigra, and the ventral tegmental area (Stahl, 2017), regulate cognition, mood, and motivation, and are responsible for schizophrenic-like symptoms (Maramai, et al., 2017). In addition, Parkinson's disease results from a loss of DA containing neurons in the nigrostriatal pathway (Graumann et al., 2002). Thus, studying DA receptors is crucial for understanding neuropsychiatric disorders related to DA system functioning.

CHAPTER TWO

DOPAMINE MEDIATED BEHAVIORS IN ADULT RATS

Behavioral Effects of Direct D1 Receptor Agonists

Agonist and antagonist drugs are useful tools for understanding the role DA receptor subtypes play in behavior (Keeler et al., 2014). For example, systemic administration of the partial D1 receptor agonist, SKF 38393, induces oral movements, grooming and locomotor activity in adult rats and mice (Desai, Terry, & Katz, 2005; Neisewander, Ong, & McGonigle, 1995). Administration of the full D1 receptor agonist SKF 82958 also increases locomotor activity in adult rats (Charntikov et al., 2011). The anatomical locus of these effects includes the NAc, because microinjecting SKF 38393 into this structure induces locomotor activity in adult rats (Charntikov et al., 2011; Meyer, 1993; Neisewander et al., 1995).

Behavioral Effects of Direct D2 Receptor Agonists

Systemic administration of selective D2 receptor agonists, such as quinpirole or bromocriptine, typically increases locomotion in rats and mice (Hoffman & Wise, 1992; Wise & Carlezon, 1994); however, treatment with low doses of a direct D2 receptor agonist actually inhibits locomotor activity (Bradbury, Cannon, Costall, & Naylor, 1984). The reason for this biphasic effect is interesting, since low doses of quinpirole stimulate autoreceptors, thereby reducing DA synthesis and/or release and, as a consequence, decrease

locomotor activity (Eilam, Golani, & Szechtman, 1989). At high doses, quinpirole directly stimulates postsynaptic receptors and produces locomotor activity (Eilam & Szechtman, 1989; Neisewander et al., 1995).

Effects of Combined Direct D1 and D2 Receptor Stimulation

Although stimulating D1 or D2 receptors alone induces locomotor activity and other behaviors, combined stimulation of D1 and D2 receptors causes a unique pattern of behavior (for a review, see Arnt, 1987). For example, systemic administration of both SKF 38393 and quinpirole, at low doses, induces locomotor activity and some rearing behaviors (Ikemoto, 2002). At high doses, co-administration of SKF 39383 and quinpirole causes more intense behaviors, including head-down sniffing, gnawing, biting, and other stereotypies (Ikemoto, 2002). Microinjecting both D1 and D2 agonists into the caudate-putamen (CPu) induces intense stereotypies, but no locomotion (Bordi & Meller, 1989; Breese et al., 1987; Waszczak, Martin, Finlay, Zahr, & Stellar, 2002).

Behavioral Effects of Indirect DA Agonists

D1 and D2 receptors play an important role in the locomotor activating effects of psychomotor stimulants, such as cocaine and amphetamine (Barret, Miller, Dohrmann, & Caine, 2004; Cabib, Castellano, Cestari, Filibeck, & Puglisi-Alegra, 1991; Dias, Carey, & Carrera, 2006). When cocaine is acutely administered, DA transporters located on the presynaptic membrane are inhibited, which results in an excess of DA in the synaptic cleft (Cabib et al.,

1991; Henry, Hu, & White, 1998). This excess DA stimulates D1 and D2 receptors and induces behavioral effects. When administered at low to moderate doses, cocaine increases the locomotor activity of adult rats (Kita, Shiratani, Takenouchi, Fukuzako, & Takigawa, 1999). Not surprisingly, when administered at high doses, cocaine causes intense stereotypies (Kita et al., 1999). Although sometimes working through different mechanisms (e.g., altering DA release characteristics), other psychomotor stimulants (e.g., amphetamine) also stimulate locomotor activity and stereotypies by increasing extracellular DA levels (Wise & Carlezon, 1994).

Behavioral Effects of Direct D1 Receptor Antagonists

Systemic administration of SCH 23390 blocks quinpirole-induced behaviors like sniffing, yawning, and locomotor activity (McDougall, Crawford, & Nonneman, 1992). The fact that a D1 receptor antagonist (SCH 23390) blocks the behavioral effects of a D2 receptor agonist (quinpirole) suggests that stimulation of the D1 receptor is crucial for the expression of D2 receptor-mediated behaviors (Delfs & Kelley, 1990). Some of these effects are mediated by the NAc, since microinjecting SCH 23390 into the NAc significantly reduces the behavioral effects of quinpirole (Bordi & Meller, 1989). Likewise, infusing SCH 23390 into the ventral lateral striatum decreases the oral behaviors, but not the locomotor activity, of amphetamine-treated rats (Delfs & Kelley, 1990). The latter result suggests that D1 and D2 receptors in the ventral lateral striatum mediate stereotypy and not locomotion.

Behavioral Effects of Direct D2 Receptor Antagonists

Not surprisingly, D2 receptor antagonists block the behavioral effects caused by a D2 receptor agonist. For example, systemic administration of sulpride significantly reduces the behavioral effects caused by microinjecting quinpirole into the NAc (Bordi & Meller, 1989; McDougall et al., 1992). Systemic treatment with a D2 antagonist also blocks the grooming behavior caused by SKF 39393 administration (McDougall et al., 1992). Thus, a functional D2 system appears necessary for the expression of D1-mediated behaviors.

CHAPTER THREE

DOPAMINE-MEDIATED BEHAVIORS IN RAT PUPS

Changes in catecholamine levels lead to a higher probability of psychiatric disorders, addiction, and motoric problems (Antonopoulos, Dori, Dinopoulos, Chiotelli, & Parnavelas, 2002). For example, early exposure to psychostimulant drugs can promote drug addiction in adulthood due to chemical changes occurring at a younger postnatal age. Byrnes and Bruno (1994) suggest that early cocaine exposure activates compensatory mechanisms that persist into adulthood and lead to addiction. In the developing brain, DA also plays a crucial role in mediating sensory motor information, such as locomotor activity and stereotypy. Drugs that act as agonists and antagonists allow us to determine DA receptor function, while microinjection techniques allow us to examine the locus of drug effects.

Behavioral Effects of Direct D1 Receptor Agonists

Systemic administration of SKF 38393, a partial D1 receptor agonist, increases probing, head lifting, and locomotion in rats at postnatal day (PD) 3 through PD 10 (Moody & Spear, 1992). At PD 20, SKF 38393 enhances grooming, oral movements, and locomotor activity (McDougall et al., 1992). Microinjecting the full D1 receptor agonist SKF 82958 directly into the CPu increases both locomotor activity and stereotypy in preweanling rats (Charntikov

et al., 2011). Lower doses of SKF 82958 preferentially induce locomotor activity, whereas higher doses increase stereotypic behaviors (Charntikov et al., 2011).

Behavioral Effects of Direct D2 Receptor Agonists

Systemic administration of quinpirole, a selective D2 agonist, increases locomotor activity, yawning, sniffing, and rearing during the preweanling period (McDougall et al., 1992). For example, quinpirole produces forward locomotion by as early as PD 3, and even greater amounts of locomotor activity at PD 10 and PD 21 (Moody & Spear, 1992). Although selective D2 receptor agonists increase locomotor activity, oral movements are inhibited by quinpirole (Der-Ghazarian et al., 2012). While most of these effects mimic what occurs in adults, D2 receptor agonists can sometimes function as antagonists in preweanling rats, but only during conditions of low dopaminergic tone (Byrnes & Bruno, 1994; Yoshida, Baella, Stuebner, Crawford, & McDougall, 2006).

Effects of Combined Direct D1 and D2 Receptor Stimulation

During early ontogeny, systemic administration of NPA, a non-selective D1/D2 receptor agonist, typically enhances stereotypy and decreases grooming, but these effects are highly dependent on dose and age (McDougall et al., 1992). At moderate doses of NPA, co-stimulation of D1 and D2 receptors increases nonstereotypic behaviors in PD 10 pups, such as wall climbing, roll curling, and forward locomotion (McDougall et al., 1992). At PD 17 and PD 20, rats also show increased locomotion after moderate doses of NPA (Der-Ghazarian et al., 2012).

In adult rats, locomotion is replaced by stereotypy when moderate or high doses of NPA are administered (Bordi, Carr, & Meller, 1989).

Indirect DA Agonist-Induced Behavior

Psychomotor stimulants, such as cocaine and amphetamine, cause an increase in locomotor activity in young rats (Anker & Carroll, 2010). Cocaine sensitivity is higher in preweanling rats (PD 5 and PD 20) than adults, perhaps due to a greater percentage of $D2^{High}$ receptors in the striatum (McDougall, Eaton, Mohd-Yusof, & Crawford, 2015). Sensitivity to cocaine begins to drop during adolescence, as the percentage of $D2^{High}$ receptors gradually declines to adult-like levels (McDougall et al., 2015).

Behavioral Effects of Direct D1 Receptor Antagonists

Systemic administration of SCH 23390, a D1 receptor antagonist, blocks the behavioral effects of the D1 receptor agonist SKF 38393 in preweanling rats (Charntikov et al., 2011). Systemic administration of SCH 23390 also blocks quinpirole-induced behaviors, such as sniffing, yawning, and locomotor activity (Chausmer & Katz, 2002; Moody & Spear, 1992; Sobrian, Jones, Varghese, & Holson, 2003), thus showing that in both preweanling and adults rats a functional D1 receptor system is necessary for the expression of D2-mediated behaviors. SCH 23390 also attenuates cocaine-induced behavioral effects in preweanling rats (Charntikov et al., 2011). These actions may be mediated by the CPu, since

microinjecting SCH 23390 into the CPu significantly reduces the behavioral impact of quinpirole in preweanling rats (Charntikov et al., 2011).

Behavioral Effects of Direct D2 Receptor Antagonists

Systemic administration of a D2 receptor antagonist (e.g., raclopride) blocks the behavioral effects of a D2 receptor agonist (e.g., quinpirole) in preweanling rats (Charntikov et al., 2011). Systemic administration of a D2 receptor antagonist also blocks the locomotor activity caused by D1 receptor agonists or cocaine (Byrnes & Bruno, 1994; Chausmer & Katz, 2002; McDougall et al., 1992). On PD 18, microinjecting SKF 82958 and quinpirole directly into the CPu increase locomotion, head down sniffing, and repetitive motor movement, while infusing raclopride into the CPu fully attenuates these agonist-induced behavioral effects (Charntikov et al., 2011).

Summary

In preweanling rats, D1 and D2 receptors interact when mediating unlearned behaviors. For example, D1 receptors are necessary for the full expression of D2-mediated locomotor activity, whereas a functional D2 receptor system is necessary for the full expression of D1-mediated behaviors (McDougall, Arnold, & Nonneman, 1990). This conclusion is not surprising since a similar relationship exists between D1 and D2 receptor systems in adult rats (Clark & White, 1987).

CHAPTER FOUR

DOPAMINE PHARMACOLOGY

Synthesis

DA, norepinephrine (NE) and epinephrine are all part of a monoamine subfamily called the catecholamines. The rate-limiting step in the production of the catecholamines is tyrosine hydroxylase. Tyrosine hydroxylase converts tyrosine into levopoda (L-DOPA). L-DOPA is then converted into the neurotransmitter DA by the enzyme dopa decarboxylase (Ugrumov, 2006).

Storage and Release

DA is stored in vesicles in the presynaptic terminal. DA exocytosis is triggered by an influx of Ca^{2+}, whereby vesicles fuse to the membrane wall and release DA into the synaptic cleft (Cooper, Bloom, & Roth, 2003).

Reuptake

Free DA in the synaptic cleft is returned to the presynaptic terminal via an ATP-dependent DA reuptake transporter (DAT) (Zanettini et al., 2018). This free-floating DA is then repackaged in vesicles for reuse. Excess DA in the presynaptic terminal is metabolized by monoamine oxidase (Zanettini et al., 2018).

Receptors

DA receptors are classified into the D1 and D2 family of receptors. The D1 family includes D_1 and D_5 receptors, whereas the D2 family of receptors contains the D_2, D_3, and D_4 subtypes (Cooper et al., 2003). Stimulating the D1 family of receptors activates the enzyme adenylyl cyclase, which increases cyclic adenosine monophosphate (c-AMP) levels (Cooper et al., 2003). Conversely, stimulating the D2 family of receptors inhibits adenylyl cyclase and reduces cAMP, thereby modulating the phosphorylation of K+ channels and inhibiting Ca^{2+} channels (Ogren, Hall, Kohler, Magnusson, & Sjostrand, 1986).

DA receptors exist in high and low affinity states. For example, DA sensitivity is associated with the proportion of D2 receptors in a high affinity state (Seeman, 2008). Work by Seeman et al. (2005) suggests that an excess of $D2^{High}$ receptors produces DA supersensitivity, which can cause DA agonists to have an exaggerated behavioral response. An excess of $D2^{High}$ receptors may be responsible for many neuropsychiatric disorders, including psychosis and drug addiction (Seeman et al., 2005).

Presynaptic vs. Postsynaptic Receptors

In addition to the various receptor subtypes, DA receptors can be differentiated according to whether they are located postsynaptically or presynaptically. DA postsynaptic receptors are G protein-coupled receptors that alter K^+ permeability (Onali, Mosca, & Olianas, 1992). For example, stimulation of

postsynaptic D1 receptors causes IPSP's in the down-stream neuron (Onali et al., 1992). In contrast, stimulation of presynaptic D2 autoreceptors decreases DA synthesis and release through G protein-mediated mechanisms (Onali et al., 1992).

Major Dopamine Pathways in the Basal Ganglia

The ventral tegmental area (VTA) is part of a motivational pathway projecting to various brain areas, such as the NAcc, hypothalamus, hippocampus and neocortex (Antonopoulos et al., 2001). The substantia nigra pars compacta (SNPC) is the starting point of another ascending pathway projecting to the CPu. Neurons projecting from the VTA and SNPC release DA at their target locations. Thus, stereotypy and motor movement are partially mediated by the nigrostriatal DA pathway projecting from the SNPC to the CPu (Bordi & Meller, 1989; Delfs & Kelley, 1990); whereas, locomotion, motivation and reward are partially mediated by the mesolimbic DA pathway projecting from the VTA to the NAcc and cortical structures (Delfs, Schreiber, & Kelley, 1990; Gong, Neil, & Justice, 1996; Meyer, Van Hartesveldt, & Potter, 1993)

Intrinsic Non-Dopaminergic Pathways in the Basal Ganglia

There are two pathways interconnecting the structures of the basal ganglia: the direct pathway and the indirect pathway (for reviews, see Alexander, DeLong, & Strick, 1986; Parent & Hazrati, 1995a,1995b). The direct pathway is formed from long axonal projections going from the CPu to the globus pallidus

internal segment (GPi) and substantia nigra pars reticulata (SNRP). The indirect pathway is comprised of a series of neurons projecting from the CPu to the globus pallidus external segment (GPe), to the subthalamic nucleus, and terminating at the GPi and SNPR. The direct pathway facilitates locomotor activity, reward, and reinforcement, while the indirect pathway suppresses the same behaviors (Dobbs et al., 2016).

CHAPTER FIVE

DOPAMINE PHARMACOLOGY: DEVELOPMENT

DA acts on D1-like and D2-like receptors to regulate motor and non-motor functions (Mishra, Singh, & Shukla, 2018). Both D1 and D2 receptors must be fully functional in order to express DA-mediated behaviors (Grigoriadis et al., 1996). As mentioned above, DA receptors are found both pre- and postsynaptically, and become functionally mature at different stages of development (Grigoriadis et al., 1996; Hedner & Lundborg, 1985; Parish, Finklestein, Drago, Borrelli, & Horne, 2001). Presynaptic DA receptors are not present at PD 4 and do not become fully mature and functional until PD 28 (Grigoriadis et al., 1996; Hedner & Lundborg, 1985). More specifically, PPP, which is a DA agonist that preferentially binds to autoreceptors, increases locomotor activity at PD 4 but decreases locomotion at PD 28, thus indicating that only postsynaptic receptors are functional at the earlier age. Indeed, D2 autoreceptors are not fully functional until the 4th postnatal week, but are neurochemically available by PD 17 (Lin & Walters, 1994).

In terms of postsynaptic D1 and D2 receptors, there is a progressive increase in the availability of striatal D1 and D2 receptors from birth through the preweanling period (Kuperstein, Eilam, & Yavin, 2008; McDougall et al., 2014; Rao, Molinoff, & Joyce, 1991; Teicher, Anderson, & Hostetter, 1995). During adolescence, there is an overproduction of D1 and D2 receptors, which gradually declines as rats enter adulthood (Andersen, 2003; McDougall et al., 2015).

In addition to these receptor changes, other DA elements develop across early ontogeny, including DAT, VMAT2, DA content, tyrosine hydroxylase activity, adenylyl cyclase activity, and D1 and D2 receptor activity (Broaddus & Bennett, 1990; Giorgi et al.,1987; Kuperstein et al., 2008; Tarazi & Baldessarini, 2000). Work done by Kuperstein et al. (2008), showed that there is a linear increase in tyrosine hydroxylase levels in the substantia nigra across ontogeny. VMAT2, DAT content, and DA levels in the CPu also exhibit a linear increase from PD 0 to PD 30 (Kuperstein et al., 2008). The ontogeny of adenylyl cyclase is similar to the ontogeny of D1 receptors, as adenylyl cyclase activity gradually increases across the preweanling period and into adolescence, and then gradually declines into adulthood (Broaddus & Bennett, 1990).

As mentioned before, DA receptor numbers increase across the preweanling period, peak in adolescence, and gradually decline into adulthood (Andersen, 2003). In terms of neuronal functionality, the ontogeny of nigrostriatal DA neurons has been examined using single-unit extracellular electrophysiological methods. Although the basal discharge rate, conduction velocity, and firing pattern of nigrostriatal neurons of 4- and 5-week-old rats was similar to adults, 2-week-old-rats differed significantly in all three of these physiological characteristics (Freeman, 2008). Specifically, the conduction velocity and basal discharge rate of nigrostriatal neurons were significantly reduced in 14-day-old rats when compared to adults. Thus, Freeman (2008)

concluded that physiological properties of nigrostriatal DA neurons are in a dynamic state of flux during the preweanling period.

CHAPTER SIX

EEDQ

N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) is an irreversible DA receptor antagonist that permanently inactivates DA receptors. EEDQ is typically used to study the time course of DA receptor replacement (Crawford, McDougall, Rowlett, & Bardo, 1992; Crawford, Rowlett, McDougall, & Bardo, 1994), but EEDQ can also be used to examine the role of DA receptors in behavior (Meller, Bordi, & Bohmarker, 1989). Although EEDQ affects a variety of receptor types, specificity can be attained by preinjecting rats with reversible selective DA antagonists (e.g., raclopride on SCH 23390) before EEDQ treatment. In this way, D1 or D2 receptors can be selectively protected from EEDQ-induced inactivation (Cameron & Crocker, 1989; Giorgi & Biggio, 1990; Meller, Bohmaker, Goldstein, & Friedhoff, 1985).

Importantly, EEDQ produces dramatically different behavioral effects in preweanling and adult rats (McDougall et al., 1992; McDougall, Crawford, & Nonneman, 1993). In adult rats, EEDQ eliminates DA agonist-induced behaviors. For example, EEDQ fully attenuated the NPA-induced locomotor activity of adult rats when measured one or two days after EEDQ administration (McDougall et al., 1992; Meller et al., 1989). Similarly, EEDQ abolishes apomorphine-induced stereotypies and quinpirole-induced rotational behavior in adult rats (Cameron & Crocker, 1989; Giorgi & Biggio, 1990). DA agonist-induced behaviors began to re-emerge when assessed four or more days after EEDQ treatment (Meller et al.,

1989).

In complete contrast, EEDQ does not block the DA agonist-induced locomotor activity of preweanling rats. For example, pretreating 17-day-old rats with EEDQ did not attenuate the acute locomotor activating effects of NPA (McDougall et al., 1992; 1993). Indeed, EEDQ often potentiates the D2 agonist-induced locomotor activity of younger animals. In preweanling rats, infusing NPA, quinpirole, or cocaine into the CPu actually stimulates greater amounts of locomotor activity in EEDQ-pretreated rats than controls (Der-Ghazarian et al., 2012, 2014). Studies using selective DA agonists, as well as D1 and D2 receptor protection, have shown that the D2 receptor is responsible for EEDQ's age-dependent behavioral effects (Der-Ghazarian et al., 2014). Thus, inactivating DA receptors with EEDQ cause a paradoxical potentiation of D2 agonist-induced locomotor activity in preweanling rats, while EEDQ blocks the DA agonist-induced behaviors of adult rats.

The reason for EEDQ's surprising age-dependent behavior effects is uncertain. The most obvious possibility is that EEDQ does not cause DA receptor loss in preweanling rats. This explanation is not correct because systemic administration of EEDQ (7.5 mg/kg) causes a substantial decline (preweanling 47%; adult 40%) in the D2 receptors of both age groups (McDougall et al., 2014). When microinjected into the CPu, EEDQ causes significantly greater reductions in the D2 receptors of preweanling rats (54%) than adult rats (35%) (Der-Ghazarian et al., 2012). Regardless, it is clear that EEDQ's ability to potentiate

the DA agonist-induced behaviors of preweanling rats cannot be explained by slight differences in the total amount of DA receptor inactivation.

Another possible explanation for EEDQ's paradoxical behavioral effects is the presence of a large D2 receptor reserve in young rats. In other words, preweanling rats may possess a large excess of unused DA receptors, thus a 50% reduction of D2 receptors, for example, would be insufficient to impact behavior. Such a D2 receptor reserve does exist in adult rats (Meller, Bohmaker, Namba, Friedhoff, & Goldstein, 1987; Meller et al., 1985; Rosengarten, Schweitzer, & Friedhoff, 1989), but there is no evidence for such a reserve in preweanling rats. For example, D2 receptors in the CPu show a linear increase across the preweanling period and into adolescence (Rao et al., 1991), thus leaving little room for a receptor reserve.

A third possible explanation is that the DA receptors remaining after EEDQ-induced inactivation exist in a high affinity state, and that these high affinity D2 receptors are significantly more prevalent in preweanling rats than adult rats. If true, these excess $D2^{High}$ receptors could be responsible for the potentiated locomotor response observed in preweanling rats (McDougall et al., 2014). Indeed, DA supersensitivity typically occurs when there is an increase in the percentage of $D2^{High}$ receptors (Seeman et al., 2005). To examine the effects of EEDQ on high affinity D2 receptors, McDougall et al. (2014) measured the $D2^{High}$ receptors of preweanling, adolescent, and adult rats one day after vehicle or EEDQ (7.5 mg/kg) pretreatment. As hypothesized, the percentage of $D2^{High}$

receptors in the CPu was significantly greater in EEDQ-treated preweanling rats than adults. Therefore, this receptor binding data is consistent with the hypothesis that EEDQ's ability to potentiate DA agonist-induced behaviors is due to an excess of $D2^{High}$ receptors.

CHAPTER SEVEN

SUMMARY AND HYPOTHESES

In summary, EEDQ fully attenuates the DA agonist-induced behaviors of adult rats. Surprisingly, preweanling rats do not respond to EEDQ in the same manner as adult rats, because EEDQ either potentiates or does not affect the DA agonist-induced behaviors of preweanling rats. The neurochemical changes that occur between the preweanling period and adolescence could be crucial to understanding the role of development in the manifestation of psychotic behaviors.

There are a number of explanations for EEDQ's age-dependent paradoxical actions. For example, EEDQ may not reduce the DA receptor levels of preweanling rats and/or preweanling rats may possess a large D2 receptor reserve. There is little evidence for these possibilities. Alternatively, EEDQ may cause a relative increase in the percentage of $D2^{High}$ receptors in preweanling rats when compared to adults. Such an effect has been observed before (McDougall et al., 2014), but it is uncertain whether this EEDQ-induced increase in the percentage of $D2^{High}$ receptors is actually capable of mediating behavioral or neurochemical effects. The purpose of this thesis was to more fully examine the latter issue.

My specific hypotheses were as follows: (1) Systemically administered EEDQ would block the cocaine-induced locomotor activity of adult rats. (2) Systemically administered EEDQ would potentiate the cocaine-induced

locomotion of preweanling rats. The latter behavioral effect was presumed to be due to an increased percentage of $D2^{High}$ receptors in preweanling rats. To test whether EEDQ does, in fact, increase D2 receptor sensitivity a GTPγS assay was conduced. GTPγS, which is a measure of receptor/G protein coupling, is one of the most common measures of D2 receptor supersensitivity (Geurts, Herman, Cumps, & Maloteaux, 1999; Seeman, Battaglia, Corti, Corsi, & Bruno, 2009). (3) I hypothesized that EEDQ would increase the E_{max} and pEC_{50} values (a measure of D2 receptor sensitivity) of preweanling rats, but not adolescent or adult rats. β-arrestin (ARRB2) and GRK6 regulate DRD_2, a super family of G protein receptors (Oda et al., 2015). When G-protein phosphorylation is triggered, GRK6 phosphorylates the receptor while ARRB2 causes internalization of the phosphorylated receptors. Importantly, ARRB2 and GRK6 levels should be depressed in situations of high D2 receptor sensitivity (Hurle, 2001; Oda et al., 2015). (4) For this reason, I hypothesized that EEDQ would decrease ARRB2 and GRK6 levels in preweanling rats, but not in adolescent or adult rats.

CHAPTER EIGHT

METHODS

Subjects

Subjects were 432 preweanling, adolescent, and adult rats. Male (N = 96) and female (N = 48) adult rats were purchased from Charles River (Hollister, CA); whereas, male (N = 96) and female (N = 48) preweanling rats, as well as male (N = 96) and female (N = 48) adolescent rats, were born and bred at California State University, San Bernardino (CSUSB). Litters were culled to 10 pups on postnatal day (PD) 3 and weaned on PD 23. Preweanling rats were kept with the dam and littermates, whereas adolescent and adult rats were group-housed with conspecifics. All rats were housed on racks in large polycarbonate maternity cages (56 × 34 × 22 cm) with wire lids. Food and water was freely available. The colony room was maintained at 22–23 °C and kept under a 12 L:12 D cycle. Subjects were cared for according to the "*Guide for the Care and Use of Laboratory Animals*" (National Research Council, 2010) under a research protocol approved by the Institutional Animal Care and Use Committee of CSUSB.

Apparatus

For preweanling (PD 18), adolescent (PD 40), and adult (PD 85) rats, behavioral testing was done in activity monitoring chambers that consist of acrylic walls, a plastic floor, and an open top (Coulbourn Instruments, Whitehall, PA).

Each chamber includes 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 Campbell, Lytle, & Fibiger, 1969; Shalaby & Spear, 1980), preweanling rats were tested in smaller chambers (26 × 26 × 41 cm) than adolescent and adult rats (41 × 41 × 41 cm).

Drugs

Cocaine was dissolved in saline; whereas, EEDQ was dissolved in a 50% DMSO solution [1:1 (v/v) in distilled water]. Drugs were purchased from Sigma (St. Louis, MO). Drugs were injected intraperitoneally (IP) at a volume of 2.5 ml/kg (preweanling rats) and 1 ml/kg (adolescent and adult rats).

Behavior Procedure

Prior to the start of behavioral testing, male and female rats were randomly assigned to treatment groups. At 24 h prior to testing (i.e., on PD 17, PD 39, or PD 84), rats were injected with vehicle or EEDQ (2.5 or 7.5 mg/kg) and then returned to their home cage. After 24 h (i.e., on PD 18, PD 40, or PD 85), rats (n = 8 per group) were taken to the experimental room and injected with saline or 15 mg/kg cocaine immediately before being placed in the testing chambers for 120 min. Locomotor activity was measured.

Homogenate [^{35}S] GTPγS Binding Assay

On PD 17, PD 39, and PD 84, male rats were injected with vehicle or

EEDQ (2.5 or 7.5 mg/kg). After 24 h, rats (n = 8 per group) were killed by rapid decapitation and CPu sections were dissected bilaterally on an ice-cold dissection plate and stored at -80 °C. On the day of assay, tissue was thawed on ice, and crude membrane homogenates 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 [^{35}S] GTPγS binding were performed in assay buffer (50 mM Tris-HCl, 120 mM NaCl) containing 30 μM GDP, 10–20 μg protein, along with DA (1 nM to 1 mM) or equivalent volumes of water. Nonspecific binding was determined in the presence of 30 μM cold GTPγS. The tubes were preincubated for 15 min at 30 °C, and then 0.1 nM [^{35}S] GTPγS was added. Following the addition of [^{35}S] 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 agonist-stimulated [^{35}S] GTPγS binding values were calculated by subtracting basal binding values (without agonist) from agonist-stimulated values (with agonist) and dividing by basal values. Agonist potency (pEC_{50}) and agonist efficacy (E_{max}) were determined by iterative

nonlinear regression fitting using Prism (Graph Pad Software).

ARRB2 and GRK6

On PD 17, PD 39, and PD 84, male rats were injected with vehicle or EEDQ (2.5 or 7.5 mg/kg). After 24 h, rats (n = 8 per group) were killed by rapid decapitation and CPu sections were dissected bilaterally on an ice-cold dissection plate and stored at -80 °C. Frozen CPu sections were weighed and sonicated into a 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 were assessed using enzyme-linked immunosorbant assay (ELISA) kits (CUSABIO, CBS-EL002135RA and CBS-EL009927RA). Briefly, various dilutions of standard ARRB2 or GRK6 were made and test samples were diluted in sample diluent (1% BSA, 0.125% Tween-20 in PBS) and added to the wells of precoated assays plates. The plates were incubated for 2 h at 37 °C. Liquid from each well was discarded and 100 µL of biotin-antibody (ARRB2 or GRK6) was added. The plates were incubated at 37 °C for 1 h. Plates were aspirated and washed three times with wash buffer (PBS, 0.1%Tween-20), after which 100 µL HRP-avidin was added to each well and the plates were again be incubated at 37 °C for 1 h. After the incubation, the plates were washed five times and TMB substrate was added to each well and incubated at 37 °C for 15-30 min in the dark. To stop the color development, stop solution was added. To

determine optical density values, absorbance readings at 450 nm, with a wavelength correction reading at 540 nm, was made using a micro plate reader (Multiskan Sky, Thermo Scientific, Waltham, MA).

Statistical Analyses

All main effects, interactions, and post hoc tests were considered significant at $p<0.05$. Litter effects were minimized by assigning no more than one rat from each litter to a particular condition (Holson & Pearce, 1992). For the behavioral experiment, distance traveled data were analyzed using a repeated measures analysis of variance (ANOVA) 3 x 3 x 2 x 12 (age x pretreatment x drug x time block). Mauchly's test was used to detect violations of the sphericity assumption. When violations of sphericity were detected the Huynh-Feldt Epsilon statistic was used to make corrections. When appropriate, Tukey tests were used for making planned and post hoc comparisons. For Tukey calculations involving time block interactions, the mean square error term was calculated using separate one- or two-way ANOVAs.

For the GTPγS binding assay a logarithmic transformation of E_{max} and pEC_{50} was conducted ($Y'=\log_{10}Y$). GTPγS (E_{max} and pEC_{50}), ARRB2, and GRK6 data were analyzed using separate 3 x 3 (age x pretreatment) between-subjects ANOVAs. Tukey tests were used for making planned and post hoc comparisons ($p<0.05$).

CHAPTER NINE

BEHAVIORAL RESULTS

Overall Analysis

Overall, female rats exhibited significantly more locomotor activity than male rats [Sex main effect, $F_{1,252}$=27.10, p<.001]. Based on this finding, the behavioral data of male and female rats were analyzed separately in the final statistical analyses.

Ontogenetic Differences in the Locomotor Activity of Male Rats (Collapsed Over Time Blocks)

Among male rats, the pre- and posttreatment factors interacted with age to affect locomotor activity [Age x Pre x Post interaction, $F_{4,126}$ = 15.24, p<.001]. In saline-treated preweanling rats, EEDQ had no effect on basal locomotor activity; however, in adolescent and adult male rats both doses of EEDQ (2.5 and 7.5 mg/kg) reduced the basal locomotor activity of saline-treated male rats (see left graph, Figure 1) [Pretreatment effects, $F_{2,21}$=18.04, p<.001, $F_{2,21}$=7.82, p<.001, and Tukey tests p<.05, respectively].

The pattern of results changed when rats were injected with cocaine. For example, preweanling male rats pretreated with 7.5 mg/kg EEDQ exhibited a potentiated locomotor response when injected with cocaine (see upper graphs, Figure 1) [Pretreatment effect, $F_{2,21}$=11.39, p<.001]. A nearly opposite effect occurred in adolescent and adult male rats, because 2.5 and 7.5 mg/kg EEDQ reduced cocaine-induced locomotor activity (see middle and lower right graphs,

Figure 1) [Pretreatment effects, $F_{2,21}$=17.59, p<.001, $F_{2,21}$=91.30, p<.001, and Tukey tests p<.05, respectively].

A separate statistical analysis comparing the effects of saline and cocaine in vehicle-pretreated male rats showed that 15 mg/kg cocaine caused greater locomotor activity in adolescent and adult rats than preweanling rats [Posttreatment x Age interaction, $F_{2,42}$=10.82, p<.001, and Tukey tests, p<.05], while the basal locomotor activity of saline-treated male rats did not differ according to age (compare open bars, Figure 1).The same statistical analysis showed that cocaine significantly increased the locomotor activity of adolescent and adult rats, but not preweanling rats. Importantly, an ANOVA including only preweanling male rats indicated that 15 mg/kg cocaine did, in fact, increase the locomotor activity of the younger age group [Posttreatment effect, $F_{1,14}$ = 22.98, p<.001, and Tukey tests, p<.05].

Ontogenetic Differences in the Locomotor Activity of Male Rats (Time-Dependent Effects)

Overall, an omnibus ANOVA showed that age interacted with pretreatment, posttreatment, and time variables to affect distance traveled scores [Age × Pre × Post interaction, $F_{4,126}$ =15.24, p<0.001; [a]Age × Pre × Post × Time Block interaction, $F_{19,610}$ =1.60, p<0.05]. To further break apart the statistically significant three- and four-way interactions, separate lower order ANOVAs were conducted at each age.

Among saline-treated preweanling male rats, EEDQ did not differentially affect distance traveled scores across time blocks; however, 7.5 mg/kg EEDQ

potentiated the locomotor activity of rats treated with 15 mg/kg cocaine on time blocks 5–12 (see upper right graph, Figure 2) [^{a}Pre × Post × Time block interaction, $F_{13,265}$ =1.91, p<0.05; and Tukey tests, p<0.05]. Separate analyses of saline-treated adolescent male 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 10 and 11 (see left middle graph, Figure 2) [^{a}Pre × Time block interaction, $F_{19,204}$ =4.51, p<0.001; and Tukey tests, p<0.05]. Likewise, 2.5 and 7.5 mg/kg EEDQ decreased the distance traveled of cocaine-treated adolescent male rats on all 12 time blocks (see right middle graph, Figure 2) [^{a}Pre × Time block interaction, $F_{5,53}$ =3.31, p<0.05; and Tukey tests, p<0.05]. Similar to adolescents, 2.5 and 7.5 mg/kg EEDQ decreased the distance traveled scores of cocaine-treated adult male rats (time blocks 1–12) and saline-treated adult male rats (time blocks 1–3 and time block 7) (see lower graphs, Figure 2) [^{a}Pre × Post × Time block interaction, $F_{8,158}$ =3.67, p<0.001; and Tukey tests, p<0.05].

Comparisons across age showed that preweanling male rats in the vehicle-saline control group exhibited less locomotor activity than similarly treated adolescent (time block 1) and adult male rats (time blocks 1–3 and time block 7) (compare left graphs, Figure 2) [^{a}Age × Pre ×Time block interaction, $F_{28,351}$ =2.22, p<0.001; and Tukey tests, p<0.05]. No age differences were apparent when EEDQ-pretreated male rats were tested after an injection of saline. When injected with cocaine, vehicle-pretreated adolescent and adult male rats had greater distance traveled scores than preweanling male rats on time blocks 2–12 (compare right graphs, Figure 2) [^{a}Age × Pre ×Time block

interaction, $F_{17,261}$ =2.20, p<0.01; and Tukey tests, p<0.05]. Conversely, cocaine-treated preweanling male rats injected with 7.5 mg/kg EEDQ had greater distance traveled scores than similarly treated adult male rats on time blocks 1–12 and similarly treated adolescent male rats on time blocks 6–12 (Tukey tests, p<0.05).

Ontogenetic Differences in the Locomotor Activity of Female Rats (Collapsed Over Time Blocks)

Among female rats, the pre- and posttreatment factors interacted with age to affect locomotor activity [Age x Pre x Post interaction, $F_{4,126}$ = 6.68, p<.001]. In female preweanling rats treated with saline, EEDQ caused a significant increase in basal locomotor activity (see upper right graph, Figure 3) [Pre effect, $F_{2,21}$=16.38, p<.001, and Tukey tests, p<.05]. However, in adolescent and adult female rats, both doses of EEDQ (2.5 and 7.5 mg/kg) decreased locomotor activity of saline-treated rats (see middle and lower right graphs, Figure 3) [Pre effects, $F_{2,21}$=14.22, p<.001, $F_{2,21}$=31.44, p<.001, and Tukey tests, p<.05, respectively].

Results changed when female rats were injected with cocaine. In preweanling female rats, 7.5 mg/kg EEDQ caused a marginally significant increase in the locomotor activity of cocaine-treated rats (see upper right graph, Figure 3) [Pre effect, $F_{2,21}$=3.40, p<.052, and Tukey tests, p<.05]. However, in adolescent and adult female rats both doses of EEDQ significantly decreased cocaine-induced locomotor activity (see middle and lower right graphs, Figure 3)

[Pre effects, $F_{2,21}$=50.38, p<.001, $F_{2,23}$=22.92, p<.001, and Tukey tests, p<.05, respectively].

As was done with male rats, a separate ANOVA comparing the effects of saline and cocaine in vehicle-pretreated female rats was conducted. Adolescent and adult female rats given 15 mg/kg cocaine exhibited greater locomotor activity than preweanling female rats [Posttreatment x Age interaction, $F_{2,42}$=10.26, p<.001, and Tukey tests, p<.05]. In addition, cocaine-treated female rats of all ages exhibited significantly greater distance traveled scores than saline-treated female rats of the same age [Tukey test, p<.05].

Ontogenetic Differences in the Locomotor Activity of Female Rats (Time-Dependent Effects)

Overall, an omnibus ANOVA showed that age interacted with pretreatment, posttreatment, and time variables to affect distance traveled scores [Age × Pre × Post interaction, $F_{4,126}$ =6.68, p<0.001; [a]Age × Pre × Post × Time Block interaction, $F_{19,610}$ =1.49, p<0.05]. To further break apart the statistically significant three- and four-way interactions, separate lower order ANOVAs were conducted at each age.

Among saline-treated preweanling female rats, 7.5 mg/kg EEDQ increased locomotion on time blocks 2-8, 10 and 11 (see upper left graph, Figure 4) [[a]Pre × Time block interaction, $F_{19,204}$ =1.13, p<0.001; and Tukey tests, p<0.05]. Moreover, 7.5 mg/kg EEDQ potentiated the locomotor activity of female rats treated with 15 mg/kg cocaine on time blocks 6, 7, 9-12 (see upper right

graph, Figure 4) [[a]Pre × Post × Time block interaction, $F_{13,265}$ =3.98, p<0.05; and Tukey tests, p<0.05].

Separate analyses of saline-treated adolescent female rats indicated that 2.5 and 7.5 mg/kg EEDQ depressed locomotion on time blocks 1-4, 6, 7, 9, 10 and 12 (see left upper graph, Figure 4) [[a]Pre × Time block interaction, $F_{19,204}$ =2.58, p<0.001; and Tukey tests, p<0.05]. EEDQ at both doses (2.5 and 7.5 mg/kg) decreased the distance traveled scores of cocaine (time blocks 1–12) and saline (time blocks 1–5 and time block 7-11) treated adult female rats (see lower graphs, Figure 4) [[a]Pre × Post × Time block interaction, $F_{8,158}$ =4.31, p<0.001; and Tukey tests, p<0.05].

Comparisons across age showed that female rats in the vehicle-saline control group exhibited an increase in locomotor activity with aging increase. Adolescent (time block 1-4, 6, 7, 9, 10, and 12) and adult (time blocks 1–5 and 7-11) female rats had more locomotor activity than preweanling rats (compare left graphs, Figure 4) [[a]Pre ×Time block interaction, $F_{28,351}$ =5.13, p<0.001; and Tukey tests, p<0.05]. There were significant age differences when EEDQ (7.5 mg/kg)-pretreated female rats were tested after an injection of cocaine. Specifically, cocaine-treated preweanling rats exhibited more locomotor activity than adolescent and adults female rats on time block 6, 7, and 9-12 [[a]Pre ×Time block interaction, $F_{28,351}$ =7.41, p<0.001; and Tukey tests, p<0.05].

[^{35}S]GTPγS Binding

GTPγS binding was significantly greater in preweanling rats than adults (Table 1) [Age main effect, $F_{2,54}$=3.98, p<0.05; and Tukey tests, p<0.05]. Both doses of EEDQ (2.5 and 7.5 mg/kg) increased E_{max} values [Drug main effect, $F_{2,54}$=15.54, p<0.001; and Tukey tests, p<0.05]. The efficacy of GTPγS binding of EEDQ-treated preweanling rats was greater than either vehicle-treated preweanling rats or EEDQ-treated adult rats [Age × Pretreatment interaction, $F_{2,57}$=3.81; p<0.05, and Tukey tests, p<0.05]. EEDQ also increased E_{max} values of adolescent rats relative to same-age vehicle controls [Tukey tests, p<0.05]. pEC_{50} binding did not vary according to age or pretreatment condition (Table 2).

ARRB2 Levels

Both doses of EEDQ (2.5 and 7.5 mg/kg) reduced ARRB2 levels [Pretreatment main effect, $F_{2,86}$=5.92, p<0.05; and Tukey tests, p<0.05]. There were age differences in which AARB2 levels increased with age. Specifically, adults had the highest levels of ARRB2, preweanling rats had the lowest levels, and adolescent rats had moderate levels of ARRB2 that were significantly different from both younger and older rats [Age main effect, $F_{2,86}$=75.28, p<0.001; and Tukey tests, p<0.05]. The Age × Pretreatment interaction was not statistically significant [p=0.21].

GRK6 Levels

EEDQ did not affect GRK6 levels. Adult rats had significantly greater GRK6 levels than both of the younger age groups [Age main effect, $F_{2,86}$=72.25, p<0.001; and Tukey tests, p<0.05]

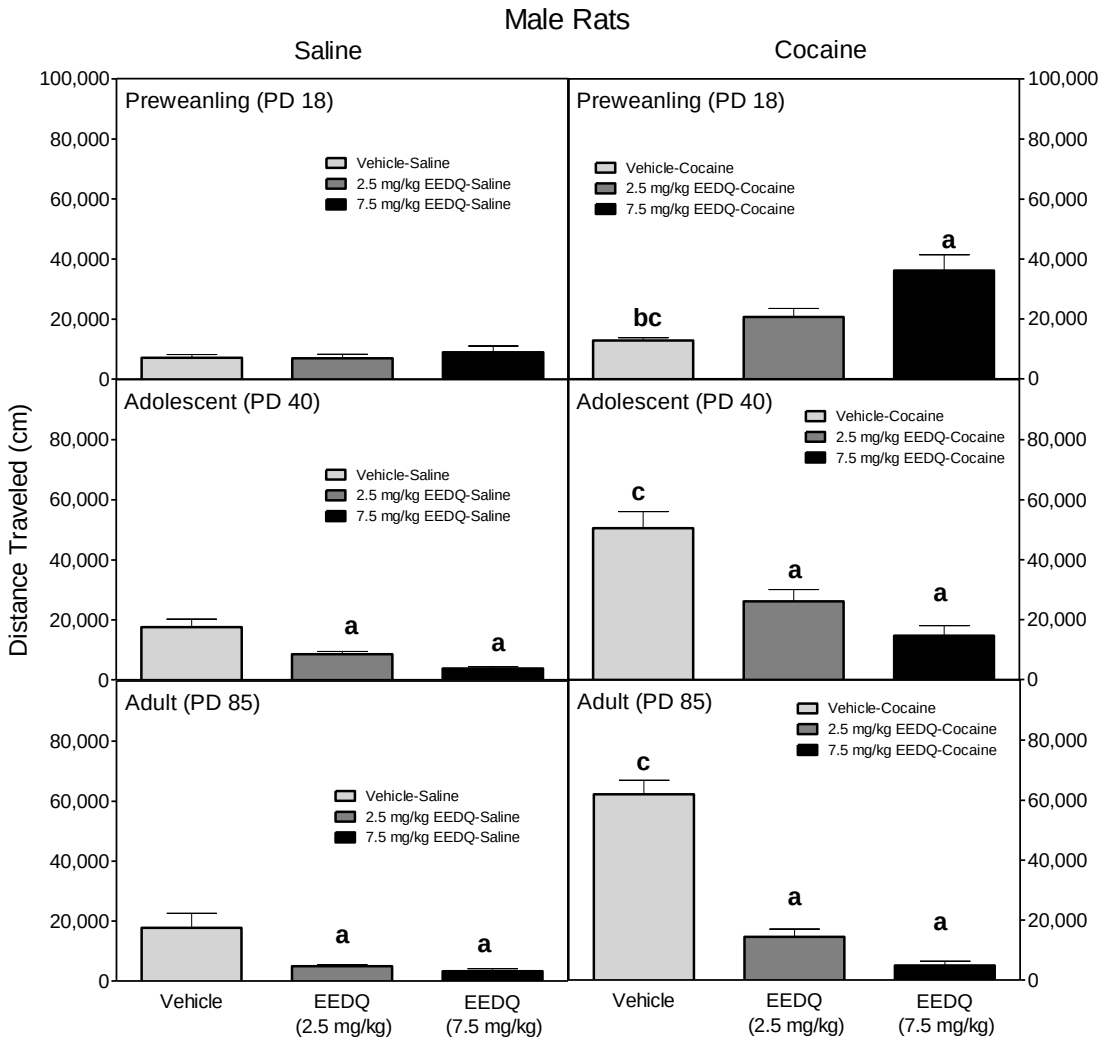

Figure 1. Ontogenetic Differences in the Locomotor Activity of Male Rats (Collapsed Over Time Blocks)

Mean (±SEM) distance traveled scores of preweanling, adolescent, and adult male rats (*n* = 8 rats per group) injected with saline or cocaine (15 mg/kg, IP) 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, IP).

'a' indicates a significant difference from vehicle-treated rats of the same age given the same post injection (i. e., saline or cocaine). 'b' indicates a significant difference from cocaine-treated adolescent and adult rats that were pretreated with vehicle. 'c' indicates a significant difference from saline-treated rats of the same age that were pretreated with vehicle.

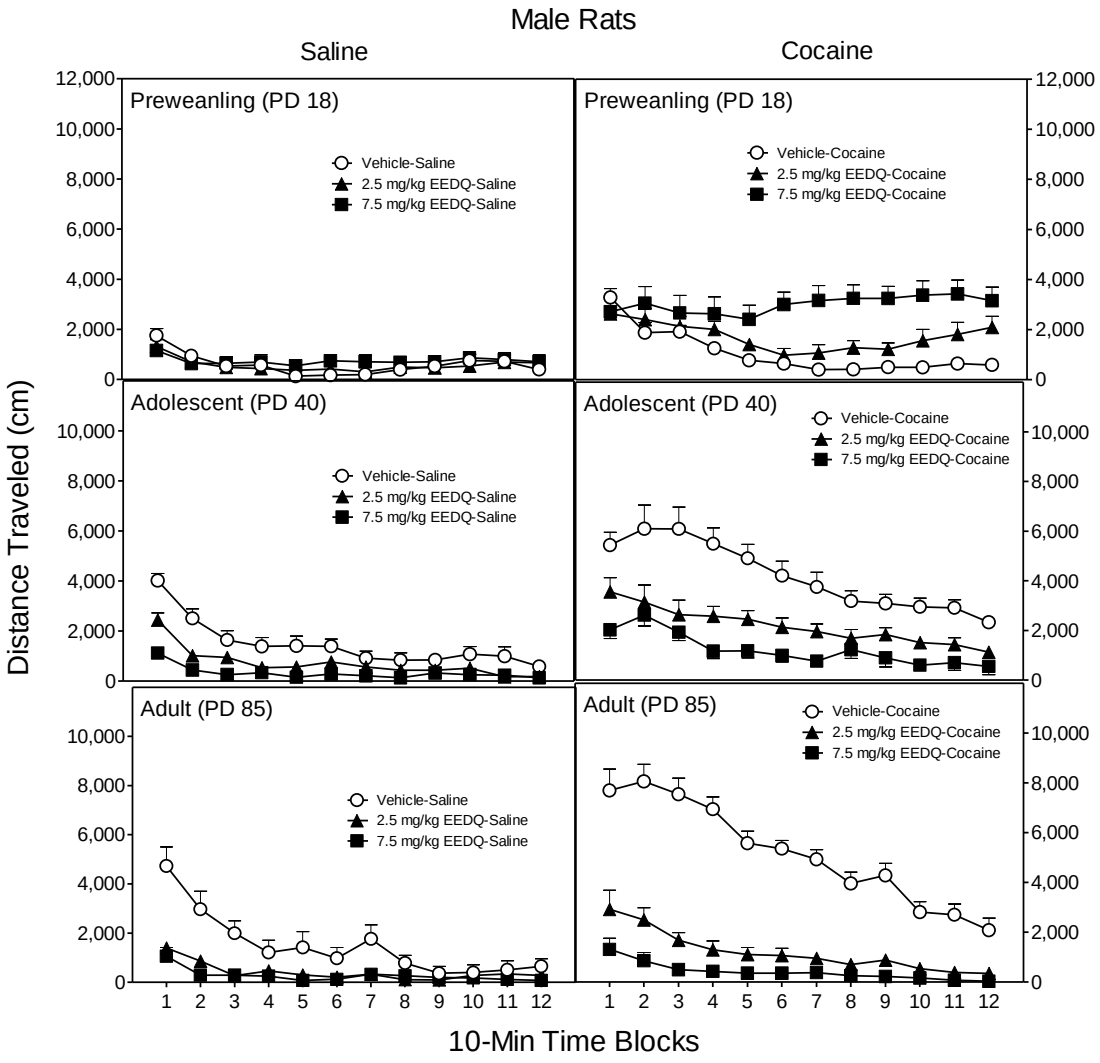

Figure 2. Ontogenetic Differences in the Locomotor Activity of Male Rats (Time-Dependent Effects)

Mean (±SEM) distance traveled scores of preweanling, adolescent, and adult male rats (*n* = 8 rats per group) injected with saline or cocaine (15 mg/kg, IP) 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, IP).

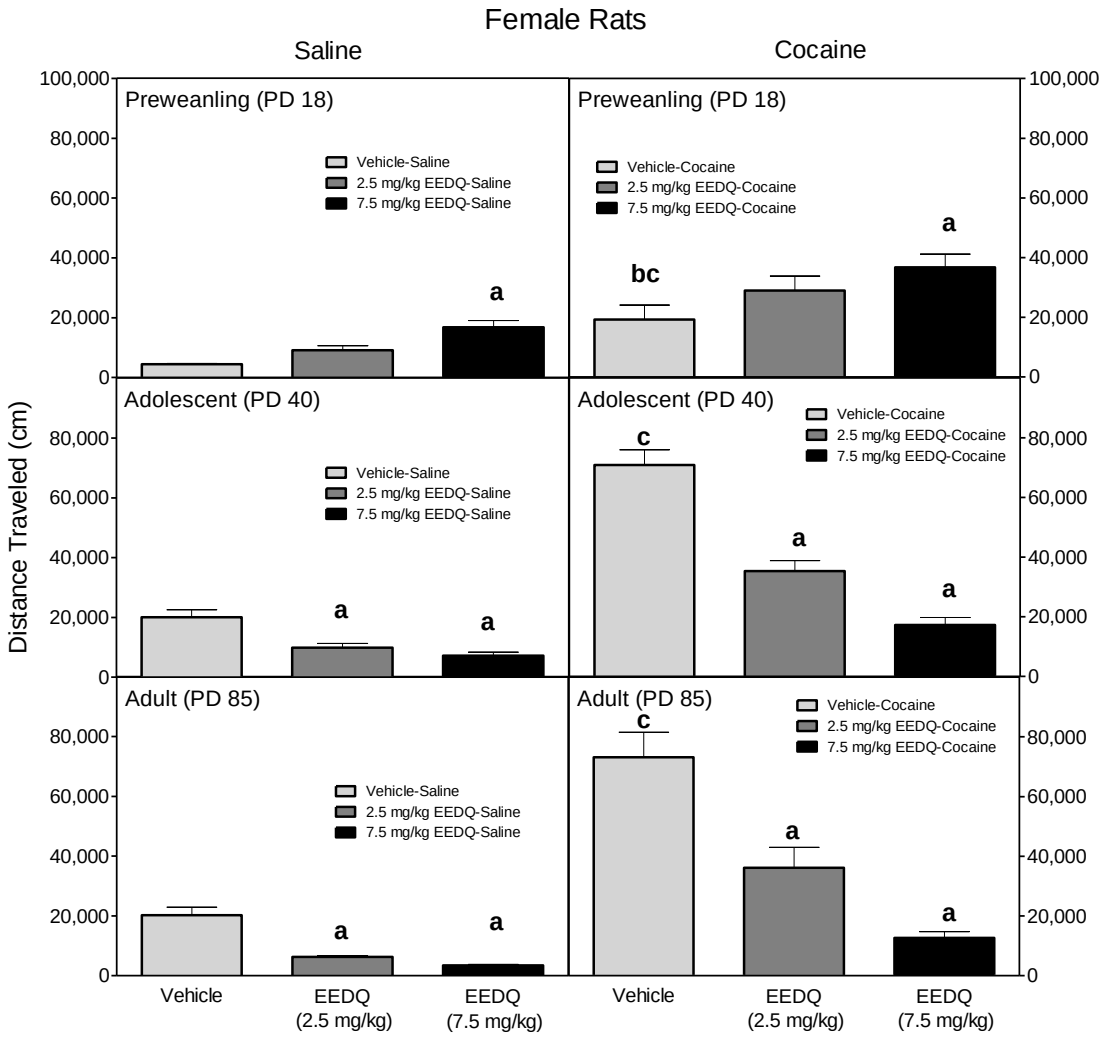

Figure 3. Ontogenetic Differences in the Locomotor Activity of Female Rats (Collapsed Over Time Blocks)

Mean (±SEM) distance traveled scores of preweanling, adolescent, and adult male rats (*n* = 8 rats per group) injected with saline or cocaine (15 mg/kg, IP) 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, IP).

'a' indicates a significant difference from vehicle-treated rats of the same age given the same post injection (i. e., saline or cocaine). 'b' indicates a significant difference from cocaine-treated adolescent and adult rats that were pretreated with vehicle. 'c' indicates a significant difference from saline-treated rats of the same age that were pretreated with vehicle.

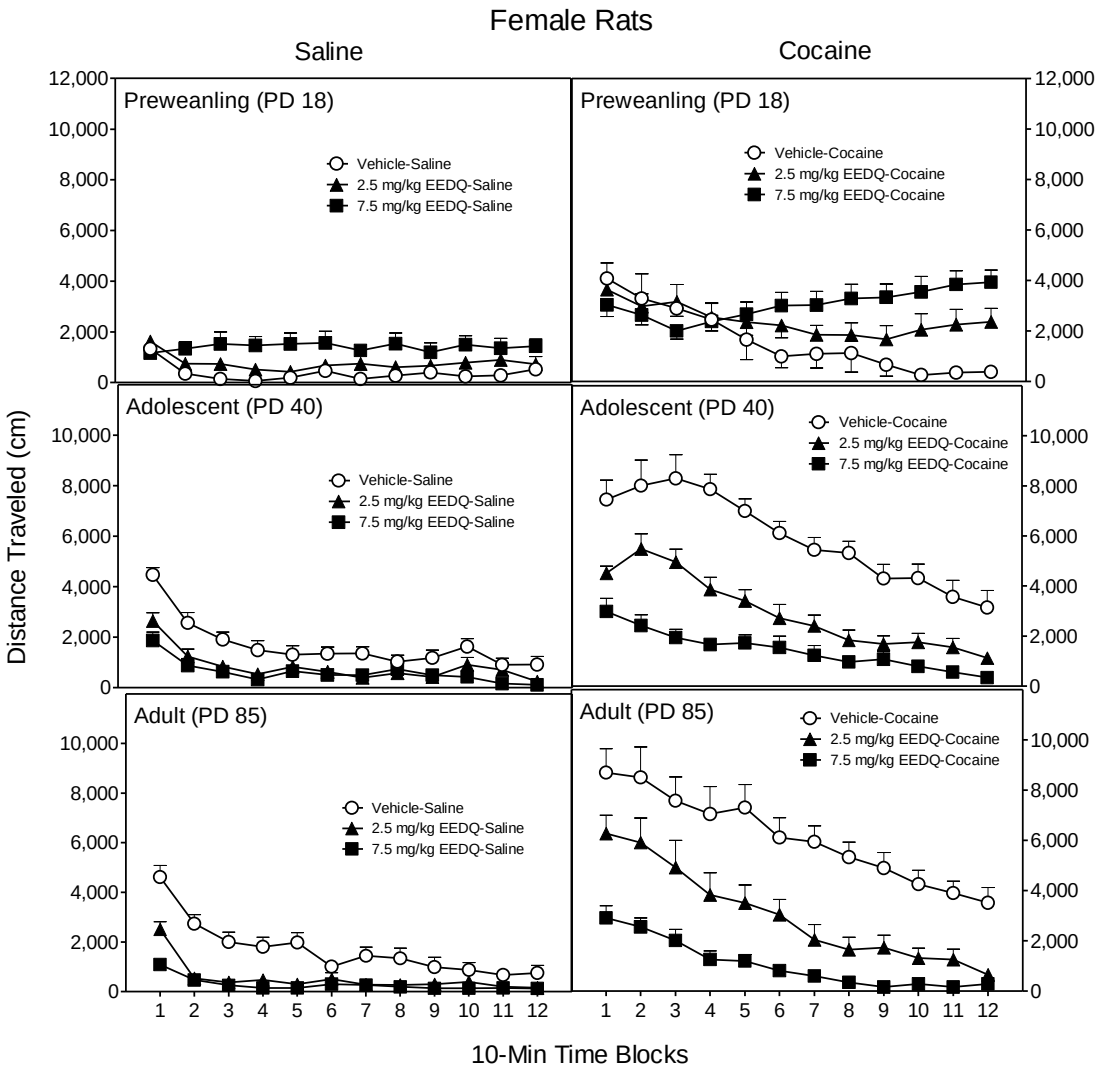

Figure 4. Ontogenetic Differences in the Locomotor Activity of Female Rats (Time-Dependent Effects)

Mean (±SEM) distance traveled scores of preweanling, adolescent, and adult female rats (*n* = 8 rats per group) injected with saline or cocaine (15 mg/kg, IP) 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, IP).

Table 1. Mean (SEM) Efficacy (E_{max}) of NPA-Stimulated [^{35}S]GTPγS Specific Binding in the Dorsal Striatum of EEDQ-Treated Preweanling, Adolescent, and Adult rats (*n* = 7 per group)

Pretreatment	Age			
	Preweanling	Adolescent	Adult	$\bar{x}$(P+A+A)
Vehicle	13.21 (2.30)	16.47 (2.76)	16.02 (1.16)	15.23 (1.24)
2.5 mg/kg EEDQ	40.76 (5.36) [cd]	28.04 (3.84) [e]	22.33 (2.92)	30.38(2.86) [b]
7.5 mg/kg EEDQ	37.12 (6.94) [cd]	29.51 (3.06) [e]	26.07 (3.71)	30.90(2.86) [b]
$\bar{x}$ (V+E+E)	30.36 (3.96) [a]	24.68 (2.21)	21.47 (1.80)	

'a' Significantly different from adult rats [Tukey tests, $p < 0.05$].

'b' Significantly different from vehicle-treated rats [Tukey tests, $p < 0.05$].

'c' Significantly different from vehicle-treated preweanling rats [Tukey tests, $p < 0.05$].

'd' Significantly different from EEDQ-treated adult rats [Tukey tests, $p < 0.05$].

'e' Significantly different from vehicle-treated adolescent rats [Tukey tests, $p < 0.05$].

Table 2. Mean (SEM) Potency (pEC_{50}) of NPA-Stimulated [^{35}S]GTPγS Specific Binding in the Dorsal Striatum of EEDQ-Treated Preweanling, Adolescent, and Adult Rats (*n* = 7 per group)

Pretreatment	Age			
	Preweanling	Adolescent	Adult	$\bar{x}$(P+A+A)
Vehicle	6.93 (0.33)	6.63 (0.51)	6.04 (0.29)	6.74 (0.18)
2.5 mg/kg EEDQ	6.54 (0.42)	6.01 (0.42)	6.97 (0.52)	6.37 (0.22)
7.5 mg/kg EEDQ	6.74 (0.13)	6.46 (0.36)	6.05 (0.42)	6.35 (0.26)
$\bar{x}$ (V+E+E)	6.53 (0.23)	6.51 (0.25)	6.42 (0.20)	

Table 3. Mean (SEM) β-Arrestin-2 Levels (pg/mg protein) in the Dorsal Striatum of EEDQ-Treated Preweanling, Adolescent, and Adult Rats (*n* = 10–12 per group)

Pretreatment	Age			
	Preweanling	Adolescent	Adult	$\bar{x}$(P+A+A)
Vehicle	577.1 (54.2)	915.2 (59.5)	1223.3 (75.9)	925.0 (60.4)
2.5 mg/kg EEDQ	476.7 (46.6)	858.7 (49.2)	1015.4 (44.8)	778.4 (49.0) [a]
7.5 mg/kg EEDQ	526.8 (54.6)	831.1 (39.0)	944.6 (33.8)	754.8 (40.3) [a]
$\bar{x}$ (V+E+E)	526.8 (30.2) [bc]	866.9 (28.2) [b]	1073.0 (39.8)	

'a' Significantly different from vehicle-treated rats [Tukey tests, $p < 0.05$].

'b' Significantly different from adult rats [Tukey tests, $p < 0.05$].

'c' Significantly different from adolescent rats [Tukey tests, $p < 0.05$].

Table 4. Mean (SEM) GRK6 Levels (pg/mg protein) in the Dorsal Striatum of EEDQ-Treated Preweanling, Adolescent, and Adult Rats (*n* = 10–12 per group)

Pretreatment	Age			
	Preweanling	Adolescent	Adult	$\bar{x}$(P+A+A)
Vehicle	342.6 (36.7)	620.9 (35.4)	758.6 (54.7)	585.6 (40.0)
2.5 mg/kg EEDQ	337.6 (19.2)	628.9 (15.2)	686.4 (46.6)	551.0 (33.0)
7.5 mg/kg EEDQ	372.6 (27.2)	624.8 (35.2)	669.7 (45.8)	546.7 (30.8)
$\bar{x}$ (V+E+E)	352.3 (16.2) [ab]	624.9 (17.0) [a]	708.3 (28.9)	

'a' Significantly different from adult rats [Tukey tests, $p < 0.05$].

'b' Significantly different from adolescent rats [Tukey tests, $p < 0.05$].

CHAPTER TEN

DISCUSSION

Behavioral Effects of EEDQ

It is well established that EEDQ attenuates locomotor activity in adult rats by reducing the amount of D2 receptors available (Der-Ghazarian et al., 2014; McDougall et al., 2015). In adolescent rats, the EEDQ-induced reduction in locomotor activity is similar to the decline observed in adult rats (McDougall et al., 2014). In preweanling rats, EEDQ also inactivates D2 receptors in the dorsal striatum; however, DA agonists cause a potentiated locomotor response (McDougall et al., 1993). An important goal of the present study was to determine what DA receptor changes are responsible for the different locomotor effects observed in EEDQ-treated rats across ontogeny.

Age-Dependent Behavioral Differences

In the present thesis, 15 mg/kg cocaine produced a statistically significant, but small, increase in the locomotor activity of PD 18 rats; however, 15 mg/kg cocaine caused a large increase in the locomotor activity of adolescent and adult rats. Regardless of age, cocaine produced a slightly greater locomotor response in female rats than male rats. Basal locomotor activity of preweanling rats was reduced relative to female and male saline-treated adolescent and adult rats.

Rats treated with saline prior to EEDQ exhibited substantially different amounts of locomotor activity depending on age. Saline-treated female

preweanling rats injected with EEDQ showed an increase in locomotor activity on the test day, while saline-treated female adolescent and adult rats injected with EEDQ exhibited a decline in locomotor activity on the test day. On the other hand, basal locomotor activity of male preweanling rats was not affected by EEDQ, even though saline-treated adolescent and adult male rats showed a significant reduction in locomotion after EEDQ treatment.

EEDQ also differentially affected cocaine-induced locomotor activity depending on age. Preweanling male rats treated with cocaine exhibited a potentiated locomotor response after EEDQ treatment. In stark contrast, adult and adolescent rats showed the opposite effect, as EEDQ attenuated cocaine-induced locomotor activity. Likewise, female preweanling rats treated with either dose of EEDQ (2.5 or 7.5 mg/kg) exhibited a potentiated locomotor response when injected with cocaine. Both doses of EEDQ reduced the cocaine-induced locomotor activity of female adolescent and adult rats.

In sum, EEDQ pretreatment (2.5 and 7.5 mg/kg) impacted locomotor behavior across ontogeny. Among preweanling rats, both doses of EEDQ increased the locomotor activity of cocaine-treated rats. Adolescent rats showed a significant reduction of locomotor activity after EEDQ treatment, although cocaine-treated adolescent rats exhibited a slight increase in distance traveled scores when compared to saline-treated rats. Adult rats showed a similar pattern of behavior as adolescent rats, since both doses of EEDQ (2.5 and 7.5 mg/kg) reduced locomotor activity in cocaine-treated adults.

Neurochemistry Results Pertaining to DA Supersensitivity

It was originally hypothesized that the increase in locomotor activity evident in EEDQ-treated preweanling rats is due to DA receptor supersensitivity. To assess this hypothesis we measured GTPγS binding, as well as ARRB2 and GRK6 content, because DA receptor supersensitivity is reliably associated with increased GTPγS binding and decreased ARRB2 and GRK6 levels (Oda et al., 2015). Moreover, it has been established that $GTP_{\gamma}S$ binding is positively correlated with the percentage of D2 receptors in a high affinity state (Seeman et al., 2009). In the present thesis, EEDQ increased the $GTP_{\gamma}S$ binding of preweanling rats, thus suggesting that EEDQ-treated rats had an excess of $D2^{High}$ receptors. Adolescent and adult rats did not exhibit a similar increase in GTPγS binding.

ARRB2 levels were also used to examine DA supersensitivity in EEDQ-treated rats, because a decrease in ARRB2 and GRK6 levels indicates supersensitivity (Hurle, 2001; Oda et al., 2015). 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 et al., 2017). In the present study, ARRB2 levels increased with age. More importantly, EEDQ-treated rats,

regardless of age, had reduced levels of ARRB2 when compared to saline-treated rats. This decline in ARRB2 levels indicated that EEDQ caused D2 receptor supersensitivity.

GRK6 levels were not significantly affected by EEDQ treatment at any age. Although it was originally hypothesized that GRK6 levels would be reduced in EEDQ-treated preweanling rats, the current results did not show such an effect. Instead, GRK6 levels were elevated in adult rats relative to the younger age groups. In sum, the EEDQ-induced increase in GTPγS binding and decrease in ARRB2 levels are consistent with a DA supersensitivity explanation, whereas the GRK6 data are not supportive.

Explanations of EEDQ-Induced Locomotor Potentiation in Preweanling Rats

The working hypothesis on which this thesis was based is that EEDQ destroys a significant percentage of D2 receptors in preweanling rats, but that the remaining D2 receptors are in a high affinity state. It is these surviving D2 receptors that we believe are responsible for the potentiated locomotor response exhibited by EEDQ-treated preweanling rats.

Alternative explanations are available for the paradoxical cocaine-induced locomotor responding of EEDQ-treated preweanling rats. First, EEDQ may not cause DA receptor loss in preweanling rats. This potential explanation is not accurate because EEDQ-treated preweanling rats exhibit a 69% reduction of dorsal striatal D2 receptors, while adults show a 80% reduction (Crawford et al., 1992; Crawford, Rowlett, McDougall, & Bardo, 1994; Der-Ghazarian et al., 2012).

Although EEDQ causes a greater reduction in the percentage of D2 receptors in adult rats, a 61% *decline* in the number of D2 receptors can certainly not explain why EEDQ *potentiates* the cocaine-induced locomotor activity of preweanling rats.

Second, these behavioral results could be explained by a large D2 receptor reserve existing in younger rats. This explanation postulates that some DA receptors were inactivated by EEDQ, but an excess of spare D2 receptors make EEDQ's receptor inactivating effects irrelevant (Der-Ghazarian et al., 2012). Although there is a D1 receptor reserve in adult rats (Meller et al.,1985,1987; Rosengarten et al., 1989) there is no evidence that a D2 receptor reserve exists in younger rats. In fact, available evidence suggests that a D2 receptor reserve is not present at any age (Meller, Goldstein, Friedhoff, & Schweitzer, 1988).

Third, we come back to our original hypothesis that in preweanling rats, and perhaps adults, EEDQ inactivates a significant number of D2 receptors, but the remaining receptors exist in a high affinity state. Consistent with this idea, EEDQ-treated preweanling rats had a greater percentage of $D2^{High}$ receptors than EEDQ-treated adolescent and adult rats. The supersensitivity explanation is further supported by results from the present thesis in which EEDQ-treated preweanling rats had increased GTPγS binding and reduced ARRB2 levels. The reason why EEDQ only potentiates the cocaine-induced locomotor activity of preweanling rats, and not adolescent and adult rats, is uncertain; however, it is

probably because EEDQ inactivates a lesser percentage of D2 receptors in younger rats (Crawford et al., 1992; Der-Ghazarian et al., 2014; Leff, Gariano, & Creese, 1984) and/or D2 receptors repopulate more quickly in younger rats than adults (Kula, George, & Baldessarini, 1992; Leff et al., 1984).

Differential Effects of EEDQ on Cocaine- and Amphetamine-Induced Locomotor Activity

An interesting aspect of the present study is that EEDQ potentiated the cocaine-induced locomotor activity of preweanling rats (Figures 1 and 3), even though we previously reported that EEDQ is unable to block amphetamine-induced locomotor activity in the same age group (Crawford, McDougall, & Bardo, 1994a). Two possibilities suggest themselves: (1) Cocaine and amphetamine have differing affinities for the DA and 5-HT transporter, and (2) cocaine and amphetamine rely on different DA pools. Cocaine has a high affinity for the DA, 5-HT, and noradrenergic transporters, while amphetamine and methamphetamine only bind to the 5-HT and noradrenergic transporters (Howell & Kimmel, 2008). This dichotomy suggests that 5-HT may contribute to the locomotor potentiation effect observed in preweanling rats. Consistent with this explanation, EEDQ inactivates 5-HT receptors (Kettle, Cheetham, Martin, Prow, & Heal, 1999), and some 5-HT receptor subtypes mediate locomotor activity (Geyer, 1996).

Perhaps of more relevance, cocaine increases extracellular DA by blocking re-uptake of DA from vesicular stores, while amphetamine primarily

relies on releasing newly synthesized DA from cytosolic pools (Kuczenski, 1983). Thus, EEDQ may only attenuate locomotor activity if the indirect DA agonist utilizes newly synthesized DA from cytosolic pools. This hypothesis is supported by a series of studies showing that EEDQ dramatically reduces the amount of DA in cytosolic pools (Crawford et al., 1992; Crawford, McDougall, & Bardo, 1994b). In sum, it is possible that the different behavioral actions of amphetamine and cocaine are a consequence of the DA pools each drug relies on.

Ontogeny of Drug Action

In a broader context, EEDQ is one of many compounds (in multiple drug classes) that produce different behavioral effects across ontogeny (Andersen, 2003, 2005; Spear, 2000). It is likely that maturational changes in DA systems, as well as other neurotransmitter systems, underlie age-dependent differences in drug action. For example, various components of the DA system, including DA content, VMAT2, and plasma membrane DA transporters, show a progressive increase from birth into adulthood (Broaddus & Bennett, 1990; Kuperstein et al., 2008). Moreover, D2 receptors increase from birth until the end of the preweanling period, are dramatically overproduced during adolescence, and then decline until adulthood age is reached (Rao et al., 1991; Teicher et al., 1995). Consistent with these maturational changes, we found that dorsal striatal ARRB2 and GRK6 levels also increase across ontogeny. Anderson and Teicher (2000) have proposed that the transient overproduction of D2 receptors may be of

clinical importance for both ADHD and schizophrenia. Our current results may also be of clinical relevance, because GRK6 and ARRB2, which are associated with $D2^{High}$ receptors, show maturational changes across early ontogeny.

Sex-Dependent Behavioral Differences

Although not a novel finding, cocaine-treated adolescent and adult female rats displayed greater locomotor activity than cocaine-treated male rats of the same age (Der-Ghazarian et al., 2012; Festa et al., 2004; McDougall et al, 2015; Schindler & Carmona, 2002). The behavioral differences between cocaine-treated males and female rats is probably not related to cocaine pharmacokinetics as peak cocaine values and cocaine half-life in brain vary minimally according to sex (McDougall et al., 2018; Schindler & Carmona, 2002). That being said, there are some active metabolites that differ between the sexes, as cocaine-treated female rats have higher levels of ecgonine methyl ester and benzoylecgonine than males (Schindler & Carmona, 2002). Whether these small differences in metabolite levels can account for sex-dependent changes in cocaine responsitivity is uncertain.

Another explanation that might account for sex-related differences in cocaine's actions involve gonadal hormones (Becker, 1999). Specifically, increased estrogen levels might be responsible for differences in cocaine sensitivity. At least two neuronal mechanisms could underlie this effect. First, estrogen inhibits GABA which, in turn, increases DA neurotransmission (Becker, 1999; Schindler & Carmona, 2002). Second, estrogen increases DA release by

down-regulating D2 receptor function (Bazzett & Becker, 1994). Males do not produce as much estrogen as females; therefore, these two estrogen-based mechanisms may explain why cocaine's behavioral effects are more pronounced in female rats (Becker, 1999).

In the present study, cocaine also caused a slight increase in the locomotor activity of female preweanling rats relative to male rats. This result was unexpected and is not consistent with many past studies showing that psychostimulants do not differentially affect the locomotor responsiveness of prepubertal male and female rats (Kozanian, Gutierrez, Mohd-Yusof, & McDougall, 2012; McDougall et al., 2015, 2018; Snyder, Katovic, & Spear, 1998). These results are also not consistent with the estrogen explanations just discussed, since the gonadal hormone levels of prepubescent rats do not differ according to sex.

Summary

DA dysfunction is responsible for many neuropsychiatric problems like anorexia, insomnia, aggravation, ADHD, Tourette's disorder, and psychosis (Kostrzewa et al., 2018; Tatsumi, Groshan, Blakely, & Richelson, 1997). Many of these disorders are prominent or are initially expressed during early ontogeny (Kostrzewa et al., 2018). Adolescence is a crucial developmental period with a unique pattern of increased DA activity in response to drugs (Spear, 2000); whereas, the late preweanling period is analogous to late childhood, which is a time that many DA-mediated disorders are first expressed (Smith & Morrell,

2008). DA supersensitivity, which was a primary focus of this study, varies across ontogeny, but appears to be especially pronounced during the preweanling period. Accumulating evidence suggests that DA supersensitivity may be related to an excess of $D2^{High}$ receptors (Seeman et al., 2009). The consequences of excess $D2^{High}$ receptors are many, as $D2^{High}$ receptors may be the critical mediating factor leading to psychosis (Seeman et al., 2005). Not only do individuals with schizophrenia have an excess of $D2^{High}$ receptors, they also show greater sensitivity to DA (Seeman et al., 2005). When considered together, the present behavioral and neurochemical results suggest that DA supersensitivity is especially pronounced during the preweanling period, which is consistent with the onset of many neuropsychiatric disorders (Tourette's disorder, ADHD, etc.).

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