Neurobiology of Stress 9 (2018) 124–132

Contents lists available at ScienceDirect

Neurobiology of Stress

journal homepage: www.elsevier.com/locate/ynstr

Stress alters social behavior and sensitivity to pharmacological activation of kappa opioid receptors in an age-specific manner in Sprague Dawley rats

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ARTICLE INFO

Keywords:
Juvenile
Adolescent
Adult
Social behavior
Kappa opioid receptor
Restraint stress
Development

ABSTRACT

The dynorphin/kappa opioid receptor (DYN/KOR) system has been identified as a primary target of stress due to behavioral effects, such as dysphoria, aversion, and anxiety-like alterations that result from activation of this system. Numerous adaptations in the DYN/KOR system have also been identified in response to stress. However, whereas most studies examining the function of the DYN/KOR system have been conducted in adult rodents, there is growing evidence suggesting that this system is ontogenetically regulated. Likewise, the outcome of exposure to stress also differs across ontogeny. Based on these developmental similarities, the objective of this study was to systematically test effects of a selective KOR agonist, U-62066, on various aspects of social behavior across ontogeny in non-stressed male and female rats as well as in males and females with a prior history of repeated exposure to restraint (90 min/day, 5 exposures). We found that the social consequences of repeated restraint differed as a function of age: juvenile stress produced substantial increases in play fighting, whereas adolescent and adult stress resulted in decreases in social investigation and social preference. The KOR agonist U-62066 dose-dependently reduced social behaviors in non-stressed adults, producing social avoidance at the highest dose tested, while younger animals displayed reduced sensitivity to this socially suppressing effect of U-62066. Interestingly, in stressed animals, the socially suppressing effects of the KOR agonist were blunted at all ages, with juveniles and adolescents exhibiting increased social preference in response to certain doses of U-62066. Taken together, these findings support the hypothesis that the DYN/KOR system changes with age and differentially responds and adapts to stress across development.

1. Introduction

The dynorphin/kappa opioid receptor (DYN/KOR) system has been identified as a potential target for the treatment of various disorders associated with stress, including anxiety, depression, and alcohol/substance use disorders (Anderson and Becker, 2017; Chavkin and Ehrich, 2014; Crowley and Kash, 2015; Knoll and Carlezon, 2010; Schwarzer, 2009; Tejeda et al., 2012; Van't Veer and Carlezon, 2013). Numerous studies in both humans and animals have demonstrated that activation of the DYN/KOR system is associated with behavioral alterations, including increased aversion, dysphoria, and anxiety, that resemble the effects of stress (Hang et al., 2015; Van't Veer and Carlezon, 2013). Additionally, blockade of DYN/KOR signaling, either through administration of KOR antagonists or through genetic or viral down-regulation/knock-down of DYN and/or KORs, attenuates stress-associated behavioral changes (McLaughlin et al., 2006; McLaughlin et al., 2003) and molecular adaptations within various brain structures known to be affected by stress (Bruchas and Chavkin, 2010; Crowley and Kash, 2015; Lemos et al., 2012).

Despite an extensive literature demonstrating dysphoric/aversive effects following activation of the DYN/KOR system, several studies have reported either opposite effects or a lack of response/sensitivity to KOR agonists (Hang et al., 2015). For example, systemic administration of KOR agonists produced anxiolytic effects on the elevated plus maze (Alexeeva et al., 2012; Braida et al., 2009; Kudryavtseva et al., 2006; Privette and Terrian, 1995). Similarly, microinjections of a KOR agonist into the infralimbic cortex also produced an anxiolytic effect (Wall and Messier, 2000b), while microinjection of the KOR antagonist, norbinaltorphimine (nor-BNI), resulted in anxiogenesis (Wall and Messier, 2000a). While these paradoxical effects of manipulations of the DYN/KOR system have been largely attributed to procedural and methodological differences across studies, a common factor that these studies

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https://doi.org/10.1016/j.ynstr.2018.09.003
Received 26 June 2018; Received in revised form 4 September 2018; Accepted 8 September 2018
Available online 11 September 2018

share is that animals were tested early in life (Diaz et al., in press). Surprisingly few studies, however, have directly compared responses to manipulations of the DYN/KOR system between younger and older animals. For instance, adolescent rats were found to be insensitive to conditioned place aversion associated with systemic administration of a KOR agonist, an effect that was observed in adult rats (Anderson et al., 2014). Another study reported that KOR activation in pre-weanlings increased appetitive responding for water (Petrov et al., 2006), opposite to what had been shown in adults (Bals-Kubik et al., 1989). Moreover, a selective KOR antagonist, nor-BNI, attenuated ethanol-induced taste aversion only in stressed adults, with stressed adolescents being insensitive to the effects of nor-BNI (Anderson et al., 2013). In support of these notably different behavioral effects associated with pharmacological activation and/or suppression of the DYN/KOR system across ontogeny, we recently found that KOR activation in the basolateral amygdala (BLA) of adolescent rats increased GABA transmission, without having an effect in the adult BLA (Przybysz et al., 2017). An age-dependent increase followed by a decrease in DYN-mediated hyperpolarization of neurons within the paraventricular nucleus of the thalamus has also been shown (Chen et al., 2015). Hence, there is clear behavioral and cellular evidence consistent with age-dependent differences in the functional role of the DYN/KOR system (Diaz et al., in press).

Although the DYN/KOR system has been demonstrated to be both engaged in and altered by stress, resulting in enhanced anxiety (Crowley and Kash, 2015; Schwarzer, 2009; Tejeda et al., 2012; Van't Veer and Carlezon, 2013), the influence of age and sex has not been carefully examined. Anxiety-like behavior in rats has been extensively assessed using the social interaction test [see (File and Seth, 2003) for references and review]. In the conventional social interaction test, two rats are placed into a testing arena, and overall time spent in social interactions is generally used as a dependent measure (File and Hyde, 1978). However, this approach combines together the discrete behavioral acts (e.g., sniffing of a partner, social grooming, following, chasing, pinning, etc.) that reflect behaviorally distinctive and differentially regulated forms of social behavior, including social investigation and play fighting. These social behaviors are characterized by distinguishable developmental patterns (Vanderschuren et al., 1997; Varlinskaya and Spear, 2008; Varlinskaya et al., 1999) and differential responsiveness to anxiety-provoking manipulations (Doremus-Fitzwater, Varlinskaya and Spear, 2009b). For example, play fighting – an adolescent-typical form of social behavior – shows an inverted U-shaped ontogenetic pattern, peaking around postnatal day (P) 30–35 and gradually decreasing to reach adult levels (Vanderschuren et al., 1997). Play fighting has a rewarding value and is crucial for development of the ability to express and understand intraspecific communicative signals (Vanderschuren et al., 2016; Vanderschuren et al., 1997). In contrast, social investigation increases with age, representing a more adult-typical form of social behavior (Vanderschuren et al., 1997; Varlinskaya et al., 1999). Play fighting, but not social investigation, is drastically increased by isolate housing throughout the juvenile and adolescent periods (Vanderschuren et al., 1997; Varlinskaya and Spear, 2008) as well as by juvenile stress (Varlinskaya et al., 2013). In contrast, social investigation is exclusively decreased by prior history of exposure to non-social stressors during adolescence and in adulthood (Doremus-Fitzwater et al., 2009b; Varlinskaya et al., 2010), with no changes in social investigation evident following juvenile stress (Varlinskaya et al., 2013). Together, these findings suggest that play fighting and social investigation may be differentially affected by pharmacological activation of the DYN/KOR system. Our modification of the social interaction test (Varlinskaya et al., 1999) allows an experimental animal to freely move toward or away from a non-manipulated social partner in a 2-compartment testing apparatus, thereby permitting assessment of social preference and/or avoidance in addition to measurement of the frequencies of play fighting and social investigation (Varlinskaya et al., 1999). Using this modified social interaction test, we have found decreases in social preference and/or social investigation to reflect anxiety-like alterations (Doremus-Fitzwater et al., 2009b; Morales et al., 2013; Varlinskaya et al., 2010; Varlinskaya and Spear, 2012).

Given the mounting evidence demonstrating age-related differences in vulnerability to and outcomes of stress exposure (Enoch, 2011; Romeo, 2017; Tottenham and Galvan, 2016) as well as in responsiveness to the aversive effects of the DYN/KOR system activation (Anderson et al., 2014), the present study was designed to systemically assess the effects of pharmacological activation of the DYN/KOR system on social investigation, social preference, and play fighting across ontogeny in non-stressed males and females as well as males and females with a prior history of repeated exposure to restraint.

2. Methods

2.1. Subjects

Juvenile, adolescent and adult Sprague-Dawley male and female rats bred and reared in our colony at Binghamton University were used. A total of 72 litters provided 360 male and female offspring to serve as experimental subjects and 360 to serve as partners. Animals were housed in a temperature-controlled (22 °C) vivarium, and maintained on a 12:12 h light:dark cycle (lights on at 0700 h) with ad libitum access to food (Purina rat chow) and water. Litters were culled to 10 pups (five males and five females) within 24 h after birth on P0 and reared until weaning with their mothers in standard plastic maternity cages (47.8 × 25.4 × 20.3 cm) with pine shavings as bedding material. Rats were weaned on P21 and placed into cages (50.8 × 40.6 × 20.3 cm) with their same-sex littermates (5 animals per cage). At all times, rats used in the current study were produced, maintained, and treated in accordance with the guidelines for animal care established by the National Institutes of Health, using protocols approved by the Binghamton University Institutional Animal Care and Use Committee.

2.2. Experimental design

The design was a 3 (age: juvenile, adolescent, adult) X 2 (sex) X 2 (stress condition: no stress or repeated restraint) x 5 (U62,066 dose: 0, 0.1, 0.2, 0,3, and 0.4 mg/kg) factorial, with six experimental animals tested per group. Juveniles were tested on P28, adolescents on P35, and adults were tested on P70. All animals from a given litter were assigned to the same stress condition. To avoid the possible confounding of litter with the experimental variables (Holson and Pearce, 1992; Zorrilla, 1997), no more than one subject per sex from a litter was assigned to a particular U-62066 dose/stress condition, with order of testing counterbalanced across litters.

2.3. Stressor procedures

Beginning at P24 for juveniles, at P30 for adolescents, and at P66 for adults, rats from the repeated stress group were removed from their home cage between 1000 and 1200 h and then restrained in age size-adjusted (5.08 cm diameter x 12.7 cm length for juveniles, 6.35 cm diameter x 15.24 cm length for adolescents, and 8.26 cm diameter x 20.32 cm length for adults) flat-bottom restrainers (Braintree Scientific, Braintree, MA) for 90 min. The animals in their restraints were placed in a novel standard plastic holding cage similar to a maternity cage that was located in a separate holding room away from the rooms where the animals were housed or later tested for social behavior. For animals in the stress group, this restraint procedure was repeated each day for 5 days. Animals placed in the control condition were non-manipulated throughout the 5-day stressor phase until the time of testing.

As in our previous studies (Doremus-Fitzwater, Varlinskaya and Spear, 2009a; Varlinskaya et al., 2010; Varlinskaya and Spear, 2012), restraint was used as the stressor, since this stressor is primarily

psychological in nature and does not induce physical pain or harm to the experimental subjects (Herman and Cullinan, 1997; Weinberg et al., 2007).

2.4. Drug administration

The selective kappa agonist U-62066 (Sigma-Aldrich) was dissolved in 0.9% saline vehicle and injected subcutaneously in a volume of 2 ml/kg 30 min prior to testing. Five doses of the drug were tested: 0 (saline), 0.1, 0.2, 0.3, and 0.4 mg/kg.

2.5. Social interaction apparatus

Social testing was conducted in Plexiglas test apparatuses (30 × 20 × 20 cm for juveniles and adolescents; 45 × 30 × 30 cm for adults) placed in rooms assigned for social testing only. Each test apparatus (Binghamton Plate Glass, Binghamton, NY) was divided into two compartments by a clear Plexiglas partition containing an aperture (7 × 5 cm for juveniles and adolescents; 9 × 7 for adults) to allow movement of animals between compartments (Varlinskaya et al., 1999; Varlinskaya et al., 2001). Testing chambers contained clean pine shavings. Each 10-min social interaction test session was conducted under dim light (10–15 lux) between 1000 and 1400 h, with a white noise generator used to attenuate extraneous sounds during testing. The behavior of each pair was recorded by a video camera mounted above the apparatus.

2.6. Testing procedures

As in our previous studies (Doremus-Fitzwater et al., 2009a; Varlinskaya et al., 2010; Varlinskaya and Spear, 2012), after the 90-min stressor exposure on day 5 (or upon removal from the home cage for non-stressed animals), each subject was injected with one of the five doses of U-62066. After this acute drug administration, each experimental animal was placed alone into a social testing apparatus for a 30 min pretest familiarization period. This pretest familiarization was conducted to substantially increase baseline levels of social interaction in the experimental animals during testing (File and Seth, 2003; Varlinskaya and Spear, 2002), hence making potential anxiogenic effects of the repeated stressors easier to observe. A same age and sex test partner unfamiliar with the experimental animal was then placed into the apparatus, and social interactions were recorded for 10 min. Partners were always non-manipulated (non-stressed and drug-naïve) animals who were unfamiliar with the test situation, not socially deprived prior to testing and, therefore, showed low levels of social activity [see (Varlinskaya and Spear, 2002)]. Weight differences between test subjects and their partners were minimized as much as possible, with this weight difference not exceeding 5 g for animals at P28, 10 g at P35, and 20 g at P70, with test subjects always being heavier than their partners.

2.7. Behavioral measures

The frequencies of social investigation and play fighting were analyzed from video recordings (Meaney and Stewart, 1981; Thor and Holloway, 1984; Varlinskaya and Spear, 2008) by a trained experimenter without knowledge of the experimental condition of any given animal. Social investigation was defined as the sniffing of any part of the body of the partner. Play fighting was scored as the sum of the frequencies of the following behaviors: pouncing or playful nape attack (experimental subject lunges at the partner with its forepaws extended outward); following and chasing (experimental animal rapidly pursues the partner); and pinning (the experimental subject stands over the exposed ventral area of the partner, pressing the animal against the floor). Play fighting can be distinguished from serious fighting in the laboratory rat by the target of the attack—during play fighting, snout or oral contact is directed towards the partner's nape, whereas during serious fighting the partner's rump is targeted (Pellis and Pellis, 1987). Aggressive behavior (serious fighting) was not analyzed in these experiments, since subjects did not exhibit serious attacks or threats.

Social preference/avoidance [see (Doremus-Fitzwater et al., 2009b; Varlinskaya et al., 2010; Varlinskaya and Spear, 2002, 2006; 2008, 2012; Varlinskaya et al., 1999; Varlinskaya et al., 2013)] was assessed by separately measuring the number of crossovers from one side of the apparatus to the other demonstrated by the experimental subject towards as well as away from the social partner and was indexed by means of a coefficient of preference/avoidance [coefficient (%) = (crossovers to the partner – crossovers away from the partner)/(total number of crosses both to and away from the partner) x 100]. Social preference was defined as positive values of the coefficient, while social avoidance was associated with negative values (Varlinskaya et al., 1999).

The total number of crossovers (movements between compartments through the aperture to and from the social partner) exhibited by each experimental subject was used as an index of locomotor activity in the social context (Varlinskaya et al., 1999).

2.8. Data analyses

Data for each dependent variable (play fighting, social investigation, preference coefficient, and total number of crossovers) were analyzed using separate 3 (age) X 2 (sex) X 2 (stress condition) X 5 (U62,066 dose) ANOVAs. In order to avoid inflating the possibility of type II errors on tests with at least three factors (Carmer, 1973), Fisher's planned pairwise comparison test was used to explore significant effects and interactions. Where significant interactions involving stress condition and U-62066 challenge dose were evident, drug-induced changes were assessed between U-62066-challenged animals and saline-challenged controls within each stress condition separately for each age as well as between non-stressed and stressed animals of the same age at a certain dose of U-62066. Sensitivity to the effects of U-62066 was assessed by the lowest dose that produced significant changes relative to saline within each age/stress condition.

3. Results

3.1. Social investigation

A significant Sex X Drug Dose interaction, $F(4, 300) = 2.78$, $p < 0.05$, reflected a general decrease in sensitivity of females to U-62066-induced suppression of social investigation relative to males. Significant sex differences in social investigation frequencies were evident at the doses of 0.3 (females: 43.1 ± 2.0; males: 33.1 ± 1.9) and 0.4 mg/kg (females: 35.3 ± 2.1, males: 27.2 ± 2.0), with females showing less social suppression than males. As can be seen in Fig. 1, to a large extent, these sex differences in sensitivity to the higher doses of U-62066 were driven by the older animals.

The ANOVA of social investigation also revealed a significant Age X Stress X U-62066 dose interaction, $F(8, 300) = 3.39$, $p < 0.001$. When data were collapsed across sex to examine this interaction, restraint was found to significantly suppress baseline social investigation (i.e., levels of social investigation seen after saline injection) in adolescent (Fig. 1D) and adult (Fig. 1F) animals only, with no socially suppressing effects of juvenile stress evident in juvenile animals (Fig. 1B). Assessment of age-related differences in sensitivity to the socially suppressing effects of the kappa opioid agonist in non-stressed animals revealed significant dose-dependent decreases in social investigation at all doses of U-62066 in juveniles (Fig. 1A) and adults (Fig. 1E), whereas in adolescents the minimal effective dose was 0.3 mg/kg (Fig. 1C). Stress-induced changes in sensitivity to the kappa agonist also differed as a function of age. Stressed animals became less sensitive to U-62066-induced suppression of social investigation, with this stress effect being more pronounced in adolescents (Fig. 1D) and adults (Fig. 1F) than in juveniles (Fig. 1B).

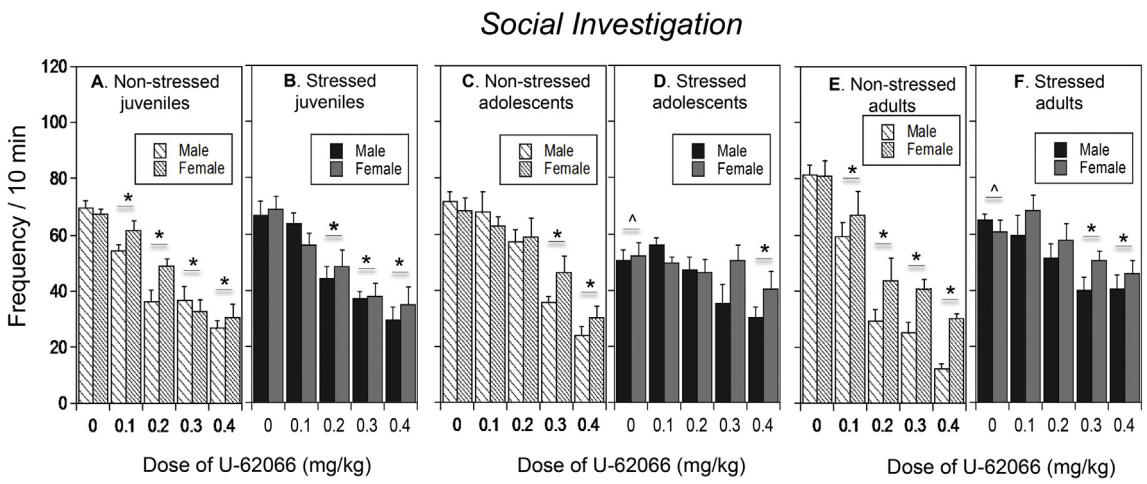

Fig. 1. Social investigation in non-stressed juvenile (A), stressed juvenile (B), non-stressed adolescent (C), stressed adolescent (D), non-stressed adult (E), stressed adult (F) male and female rats: Effects of the selective KOR agonist U-62066. Asterisks (*) denote significant drug effects within each age/stress exposure condition relative to vehicle, with data collapsed across sex. Significant stress-related changes in each age group under basal (0 mg/kg dose) conditions, with data collapsed across sex, are denoted with (^). Data are expressed as mean ± SEM, $p < 0.05$.

Specifically, in stressed juveniles, social investigation was significantly reduced at all doses except for the lowest dose of 0.1 mg/kg (Fig. 1B). In contrast, stressed adolescents became substantially less sensitive to the social suppression induced by U-62066 and responded by a significant decrease in social investigation only to the highest dose of 0.4 mg/kg (Fig. 1D). Similarly, stressed adult animals demonstrated significant suppression of social investigation at 0.3 and 0.4 mg/kg of the kappa agonist, with no significant decreases emerging at lower doses (Fig. 1F).

3.2. Social preference/avoidance

The ANOVA revealed a significant Age x Stress X U-62066 dose interaction, $F(8, 300) = 2.33$, $p < 0.05$, with no main effect or interactions involving sex evident for this measure. When data were collapsed across sex, baseline social preference again differed as a function of stress conditions in adolescent (Fig. 2D) and adult (Fig. 2F), but not juvenile (Fig. 2B), rats, with exposure to the stressor significantly decreasing baseline social preference relative to non-stressed animals among adolescents (Fig. 2C) and adults (Fig. 2E) but not among stressed juveniles (Fig. 2B). Marked age differences in sensitivity to U-62066 were evident in non-stressed animals. Non-stressed juveniles (Fig. 2A) were less sensitive to the social anxiogenic effects of U-62066 than older animals, showing significant decreases in social preference at the highest dose only, whereas non-stressed adolescents demonstrated significant decreases in social preferences at the doses of 0.3 and 0.4 mg/kg (Fig. 2C). Non-stressed adults were the most sensitive to the anxiogenic effects of U-62066, showing negative values of the coefficient at 0.2, 0.3, and 0.4 mg/kg doses, with the highest dose of this kappa agonist (0.4 mg/kg) producing substantial social avoidance in adults (Fig. 2E). These age-dependent anxiogenic effects of the selective kappa agonist were eliminated by repeated restraint, with no decreases in social preference relative to corresponding saline-injected controls evident at any age (Fig. 2B,D,F). Interestingly and in contrast, juveniles showed significant increases in social preference at 0.4 mg/kg (Fig. 2B), with a similar socially anxiolytic effect evident in adolescents at the lowest dose of 0.1 mg/kg (Fig. 2D).

3.3. Play fighting

A significant Age x Stress X U-62066 dose interaction, $F(8, 300) = 2.84$, $p < 0.01$, was evident for play fighting. Similar to social preference/avoidance, no main effect or interactions involving sex were

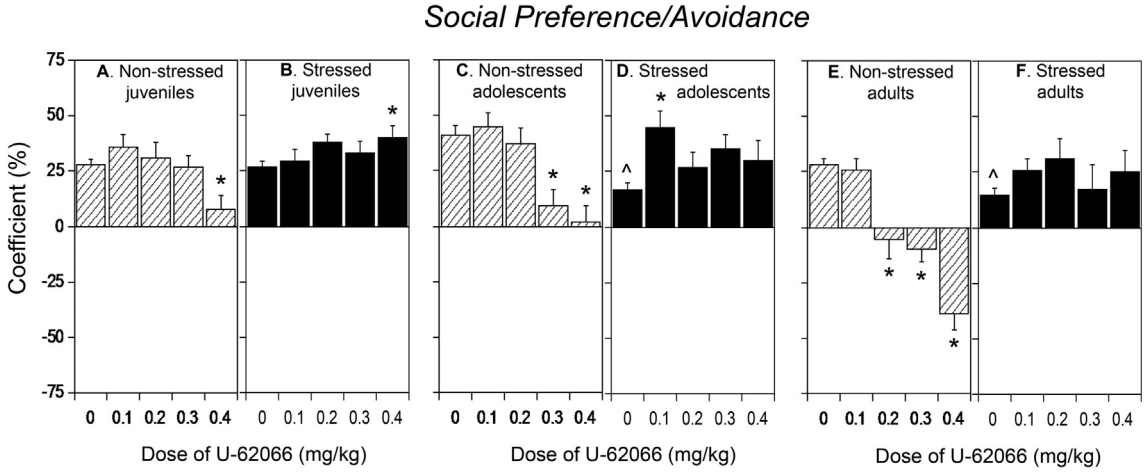

Fig. 2. Social preference/avoidance in non-stressed juvenile (A), stressed juvenile (B), non-stressed adolescent (C), stressed adolescent (D), non-stressed adult (E), and stressed adult (F) rats, with data collapsed across sex: Effects of the selective KOR agonist U-62066. Hatched bars represent data for non-stressed rats, black bars represent data for stressed animals. Asterisks (*) denote significant drug effects within each age/stress exposure condition relative to vehicle. Significant stress-related changes in each age group under basal (0 mg/kg dose) conditions are denoted with (^). Data are expressed as mean ± SEM, $p < 0.05$.

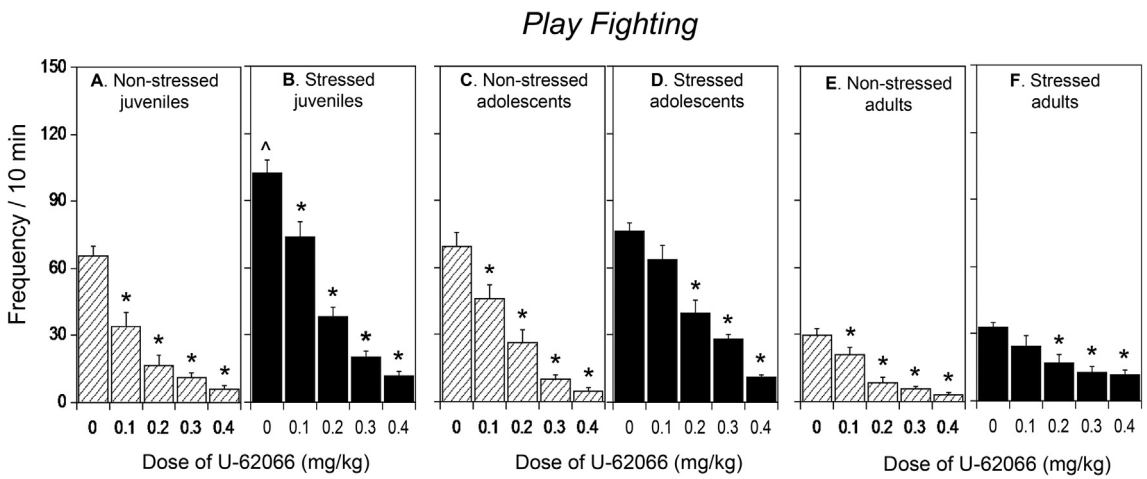

Fig. 3. Play fighting in non-stressed juvenile (A), stressed juvenile (B), non-stressed adolescent (C), stressed adolescent (D), non-stressed adult (E), and stressed adult (F) rats, with data collapsed across sex: Effects of the selective KOR agonist U-62066. Hatched bars represent data for non-stressed rats, black bars represent data for stressed animals. Asterisks (*) denote significant drug effects within each age/stress exposure condition relative to vehicle. Significant stress-related changes in each age group under basal (0 mg/kg dose) conditions are denoted with (^). Data are expressed as mean ± SEM, $p < 0.05$.

evident for this measure; therefore, data presented in Fig. 3 were collapsed across sex. The most striking effect of stressor exposure was evident in juveniles (Fig. 3A and B), with exposure to restraint markedly increasing play fighting under basal, saline challenge conditions (Fig. 3B). In non-stressed animals all four doses of U-62066 significantly suppressed play fighting regardless of age (Fig. 3A,C,E). In stressed adolescents (Fig. 3D) and adults (Fig. 3F) the suppressing effects of the lowest dose did not reach statistical significance whereas a U-62066-related decrease in play fighting was seen at all doses in stressed juveniles.

3.4. Locomotor activity (Table 1)

The analysis of general locomotor activity under social circumstances indexed via total number of crossovers between compartments revealed significant main effects of age, $F(2, 300) = 25.39$, $p < 0.0001$ and drug dose, $F(4, 300) = 143.27$, $p < 0.0001$ but no main effect or interactions involving sex and/or stress condition. In general, juveniles and adolescents demonstrated significantly more crossovers than their older counterparts, with all doses of U-62066 gradually suppressing locomotor activity under social test circumstances regardless of age or stress condition (Table 1).

4. Discussion

Given evidence that the DYN/KOR system in younger animals may function and adapt to stressors differently than in adulthood [see review by (Diaz et al., in press)], this study systematically tested the effects of a selective KOR agonist on social investigation, social preference, and play fighting in juvenile, adolescent and adult male and female rats. The effects of the selective KOR agonist were age- and stress-dependent, with sex exerting a modest effect on one response measure. In non-stressed animals, pharmacological activation of KORs suppressed social behavior at all ages, although sensitivity to this social suppression varied with age depending on the social measure under investigation. Repeated restraint exerted anxiogenic effects in adolescents and adults indexed via decreased baseline levels of social investigation and the preference/avoidance coefficient. These stressor effects were not seen in younger animals, with repeated restraint increasing play fighting in juveniles. Restraint generally made animals less sensitive to the suppressant actions of U-62066. These stress-associated consequences were particularly notable for social preference/avoidance in mature animals, with U-62066-induced social avoidance in non-stressed adults being converted to social preference following repeated restraint stress. A sex difference was evident for social investigation, with females showing reduced drug-induced suppression on this measure relative to males.

4.1. Ontogenetic social effects of U-62066 in non-stressed animals

It is well established that activation of the DYN/KOR system in adulthood results in dysphoria, aversion, and increased anxiety-like behaviors in both humans and animals (Hang et al., 2015; Schwarzer, 2009; Van't Veer and Carlezon, 2013). However, several studies have reported opposite effects or an insensitivity to KOR activation early in ontogeny (Diaz et al., in press). For instance, neonates demonstrated increased appetitive responding to a surrogate nipple providing water following administration of a KOR agonist (Petrov et al., 2006). In a

Table 1
General locomotor activity under social circumstances indexed via total number of crossovers between compartments in juvenile, adolescent and adult non-stressed and stressed males and females following administration of the selective kappa opioid agonist U-62066.

Age (postnatal day) at test	Stress Condition	Dose of U-62066 (mg/kg)				
		0	0.1	0.2	0.3	0.4
P28	Non-stressed	46.1 ± 2.1	30.1 ± 3.4	23.6 ± 2.2	20.1 ± 2.3	14.1 ± 1.8
	Stressed	48.2 ± 1.5	36.0 ± 3.2	27.4 ± 1.9	19.6 ± 2.4	14.3 ± 1.8
P35	Non-stressed	45.7 ± 3.1	28.7 ± 2.3	23.6 ± 2.9	15.3 ± 2.1	10.0 ± 1.2
	Stressed	39.9 ± 2.9	28.8 ± 2.6	23.7 ± 3.4	15.7 ± 2.6	13.2 ± 1.1
P70	Non-stressed	35.5 ± 3.8	22.2 ± 2.1	14.1 ± 1.9	12.6 ± 1,4	9.9 ± 1.9
	Stressed	35.6 ± 1.7	24.9 ± 2.4	19.9 ± 2.1	14.5 ± 1.9	14.6 ± 1.9

Data shown as mean ± standard error of the mean.

conditioned place preference/aversion paradigm, adolescents were insensitive to KOR activation whereas adults showed conditioned place aversion to the same doses of the KOR agonist (Anderson et al., 2014). Several studies have also reported anxiolytic effects of KOR agonists on the elevated plus-maze in rats that were tested at a weight consistent with adolescence (~150–210 g) (Alexeeva et al., 2012; Braida et al., 2009; Privette and Terrian, 1995). Consistent with these behavioral observations, adolescents in the present study were found to be less sensitive to the reductions in social investigation induced by U-62066 relative to both juveniles and adults. When social preference was the measure under investigation, a gradual ontogenetic increase in responsiveness to U-62066 was evident with juvenile animals being the least sensitive and adults being the most sensitive to U-62066-induced decreases in social preference. Adults not only demonstrated significant reductions in social preference at lower doses, but also exhibited social avoidance at the highest dose (0.4 mg/kg), indicating that this dose was severely anxiogenic to that age group [see (Varlinskaya et al., 2010)]. Overall, these findings complement and add to the growing literature demonstrating that younger animals are less sensitive to the dysphoric, aversive, and anxiogenic effects of KOR agonists than their more mature counterparts.

While the mechanism(s) underlying this early-life insensitivity are unknown, we previously found that activation of KORs in the BLA (but not CeA) increases GABA transmission in adolescent males, but not in adult males, while not affecting glutamate transmission at either age (Przybysz et al., 2017). As a result, excitability of the BLA would be reduced in adolescents relative to adults – findings similar to that which has been reported following KOR activation in adolescent animals (Huge et al., 2009). Based on the role of the BLA, we would predict that these physiological effects of KOR activation in adolescents would likely produce anxiolytic effects. Age-dependent changes in KOR-induced outward currents have also been reported in the paraventricular nucleus of the thalamus, a brain structure that interfaces with the anxiety circuit, with the magnitude of these currents increasing between 2 and 4 weeks of age, followed by a reduction by 8 weeks of age (Chen et al., 2015). Studies to examine how these various ontogenetic differences in KOR function in anxiety-relevant brain regions contribute to the ontogenetic differences in KOR stimulation observed in this study are warranted and are currently underway.

4.2. Stress differentially affects social behavior across ontogeny

Substantial evidence has accumulated that consequences of repeated exposure to stressors early in life may be more detrimental than later stress exposure due, in part, to stress-induced alterations in critical neurodevelopmental processes (Romeo, 2017; Tottenham and Galvan, 2016). Consistent with this, in our previous studies, adolescents and adults showed similar patterns of social alterations associated with repeated restraint (Varlinskaya et al., 2010), whereas juveniles differed drastically from older animals (Varlinskaya et al., 2013). Similarly, in the present study, adolescents and adults demonstrated significant stress-related decreases in social investigation and social preference that were not evident in juveniles. Instead, juveniles responded to the prior stress exposure with an enhancement of play fighting – a form of social behavior that is more expressed in juveniles and adolescents than in adults (Vanderschuren et al., 1997; Varlinskaya et al., 1999). These observed stress-associated alterations were not related to overall locomotor activity indexed via total number of crossovers, with crossovers being unaffected by stress at all ages. It is not the case that juveniles were unable to respond to stressors given that juvenile rats as well as their more mature counterparts have been shown to respond similarly to a novel, anxiety-provoking test situation by an enhancement in social anxiety-like behavior, indexed by a transformation of social preference into social avoidance (Varlinskaya and Spear, 2006, 2008). One possible contributor to the juvenile-specific response to restraint stress is that the 90-min periods of restraint were perceived by juveniles as significant social deprivation, with this social isolation producing substantial increases in play fighting immediately thereafter (Varlinskaya and Spear, 2008). Timing of assessment following stress is clearly an important aspect to consider, since different adaptations occur in the DYN/KOR system over time (Knoll and Carlezon, 2010). Studies examining these time-course-dependent adaptations across ontogeny are ongoing in our lab and will shed light on these factors.

The differential responses of juvenile animals and their older counterparts in the present study could potentially be related to pubertal status, with some research showing pre-pubertal animals to differ markedly in their responsiveness to stress from post-pubertal, adult rats (Koenig et al., 2011; McCormick et al., 2007; Romeo, 2010). For instance, pre-pubertal stress has been shown to enhance anxiety-like behavior and substantially reduced exploratory behavior in adulthood (Jacobson-Pick and Richter-Levin, 2010; Tsoory et al., 2007). However, when these young animals were tested immediately after exposure to stressors, they demonstrated increases in exploratory behavior (Horovitz et al., 2012) and decreases in anxiety (Jacobson-Pick and Richter-Levin, 2012), findings reminiscent of the results of the present study. Physical and hormonal signs of puberty are observed earlier in female than male rats, with pubertal maturation generally completed by P36 in Sprague-Dawley females and by P44 in Sprague-Dawley males (Vetter-O'Hagen and Spear, 2012). Yet, in the present study, the effects of the stressor on social investigation and social preference did not differ at P35 between male rats (who were prepubertal at that age) and female rats (that were reaching the end of pubertal maturation), with P35 rats of both sexes showing adult-like social responding to repeated restraint. These findings are consistent with earlier work finding similar stress-induced alterations in the social consequences of repeated stress in adolescent and adult males and females (Doremus-Fitzwater et al., 2009b; Varlinskaya et al., 2010). Collectively these data support the notion that the observed age-dependent differences between juveniles and adolescents in the social consequences of repeated restraint may not be a function of pubertal maturation but rather may be associated with age-related neurobiological differences (Spear, 2000, 2011). Clearly more studies are required for better understanding of immediate and long-lasting consequences of juvenile versus adolescent stress.

4.3. Stress differentially alters sensitivity to KOR activation across ontogeny

One of the primary objectives of this study was to determine how stress alters the effects of pharmacological activation of KORs across ontogeny. In general, stress diminished responses to U-62066, with these stress-associated effects being least pronounced among juveniles, and most marked in adults. Specifically, doses of the KOR agonist that produced robust reductions in social investigation and social preference in non-stressed adolescents and adults no longer produced those effects following restraint. In adults, the social anxiogenic effect of U-62066 (indexed via significant reductions in social preference) was eliminated by stress, whereas in adolescents, the lowest dose of U-62066 (0.1 mg/kg) reversed the socially anxiogenic effect of stress, demonstrating a potentially anxiolytic effect of KOR activation at this low dose. A similar anxiolytic effect of the highest dose of U-62066 (0.4 mg/kg) was evident in stressed juveniles, with this dose significantly increasing social preference relative to vehicle. The mechanisms driving the stress-induced insensitivity to the suppressant effects of U-62066 on social investigation and social preference (see Figs. 1 and 2) have yet to be determined. One possibility is that this stress-induced drug insensitivity is a result of the DYN/KOR system already being activated by the stressor and potentially saturated at the time of testing, given that testing was initiated almost immediately following the final restraint. Alternatively, there is evidence that KORs can become desensitized following exposure to stress (McLaughlin et al., 2004), resulting in a decreased sensitivity to administration of a KOR agonist reminiscent of that found in stressed animals in the present study.

Other studies have reported anxiolytic effects of KOR agonists in younger animals. Interestingly, studies reporting such anxiolytic effects have used animals that were likely or confirmed to have been ordered and shipped from an animal vendor (Alexeeva et al., 2012; Braida et al., 2009; Privette and Terrian, 1995). There is compelling evidence that shipping causes major stress in animals, which can be mimicked by adolescent stressors such as social isolation that can produce numerous behavioral and physiological alterations (Chappell et al., 2013; Rau et al., 2015). Therefore, these data suggest that early-life stress can result in adaptations in the DYN/KOR system that differ from those seen in the adult DYN/KOR system. Studies investigating the underlying mechanisms are needed to better understand age-dependent neuroadaptations that occur in the DYN/KOR system following stressor exposure.

In the present study, we tested the effects of U-62066 immediately following the final exposure to restraint stress. However, it would also be important to examine whether stress-induced alterations in responsiveness to KOR activation can be observed at a later time point, and more importantly, whether these long-term alterations differ in animals exposed to restraint as juveniles, adolescents, or adults. Recent reports have investigated this very question in adults and have found somewhat contrasting results. Laman-Maharg et al., (2017) found that adult male California mice continued to exhibit aversive responding to a KOR agonist 24 h following exposure to social defeat stress, whereas stressed females showed a blunted response (Laman-Maharg et al., 2017). However, a study by Al-Hasani et al. (2013) found that acute forced swim stress potentiated a subsequent KOR-mediated reinstatement of cocaine conditioned place preference in adult male C57BL/6 mice, whereas a chronic mild stress paradigm reduced KOR-induced reinstatement of cocaine conditioned place preference (Al-Hasani et al., 2013). These studies highlight the complexity of the DYN/KOR system and the numerous neuroadaptations that can ensue depending on various experimental variables, including age at the time of stress exposure, stressor chronicity as well as biological variables such as sex.

4.4. Age, stress and DYN/KOR system's roles in play fighting

Adolescent and adult animals demonstrated substantial reductions in sensitivity to effects of U-62066 on social preference, and to some extent, social investigation – two social behaviors previously shown to be stressor-sensitive (Varlinskaya et al., 2010; Varlinskaya et al., 2013). Yet, more limited stress-associated changes in sensitivity to U-62066 were evident in all age groups when play fighting was the measure under investigation. In general, play fighting demonstrated by juvenile and adolescent rats is considered to be under control of the endogenous opioid systems (Trezza et al., 2010). Pharmacological activation of mu opioid receptors enhances play fighting in juvenile and adolescent rats [reviewed in (Trezza et al., 2010)], whereas pharmacological activation of KORs decreases social play behavior (Vanderschuren et al., 1995). The results of the present study are in agreement with these early findings in that the selective KOR agonist effectively and dose-dependently decreased play fighting in adolescent and adult animals. However, it seems unlikely that the endogenous DYN/KOR system is involved in modulation of play fighting in previously stressed animals, given that no baseline decreases in play fighting were seen in saline control animals in response to stress-induced activation of this system. In contrast, stress exposure substantially increased play fighting in juveniles. Taken together with the age-specific effects of repeated restraint on social investigation and social preference, it is likely that the endogenous DYN/KOR system plays little role, if any, in modulation of play fighting in previously stressed animals.

4.5. Sex differences in DYN/KOR system function

Sex differences are prevalent in many neuropsychiatric disorders, with, for instance, females being more vulnerable than males to psychopathologies like anxiety and depression (Kessler, 2003). Importantly, increased susceptibility to psychopathologies may contribute to the increased negative consequences of the use and abuse of drugs, including alcohol (Becker et al., 2012). Given evidence for a stimulatory role of the DYN/KOR system in negative affect and affective disorders (Knoll and Carlezon, 2010; Tejeda et al., 2012; Van't Veer and Carlezon, 2013), it might be expected that the DYN/KOR system would be more sensitive in females than males. However, the few studies that have examined sex differences in responsivity of the DYN/KOR system have generally found females to be less sensitive than males to both activation and inhibition of the DYN/KOR system, both in terms of changes in pain response and affect [see reviews by (Becker and Chartoff, 2018; Chartoff and Mavrikaki, 2015; Williams and Trainor, 2018)]. While the underlying mechanisms driving these sex differences are not well understood, a number of differences have been reported regarding the role of the DYN/KOR system in females, including polymorphisms in the prodynorphin (pDYN) gene, interactions with the melanocortin 1 receptor (MC1R) at the genetic level, heterodimerization of KORs and mu opioid receptors (MORs), and unique interactions with gonadal hormones (Chartoff and Mavrikaki, 2015; Chartoff et al., 2009). Only modest sex differences were evident in the current study, with females showing reduced sensitivity to systemic administration of U-62066 relative to males only in terms of social investigation, an effect evident at the higher (0.3 and 0.4 mg/kg) doses of the drug. Although not interacting significantly with age, this subtle sex difference appeared to be more robust in the older, post-pubertal animals.

5. Conclusions

Responsiveness to repeated restraint stress in terms of both stress-induced behavioral alterations and stress-associated changes in the social consequences of activation of the DYN/KOR system differs notably in juveniles relative to adolescents and adults. Consistent with our previous findings, stress-induced suppression of social investigation and social preference were evident in adolescents and adults, but not juveniles, with this youngest age group instead demonstrating substantial stress-induced increases in play fighting (Doremus-Fitzwater et al., 2009b; Doremus-Fitzwater et al., 2010). Furthermore, more robust stress-associated decreases in responsiveness to pharmacological KOR activation were seen in adults, and to some extent adolescents, than juveniles, suggesting ontogenetic increases in stress-induced activation of the DYN/KOR system. Stressed juveniles and adolescents exhibited increases in social preference to KOR activation, suggesting that normal anxiogenic effects of KOR stimulation are reversed by stress in animals at these ages. Sex differences in responsiveness to pharmacological activation of KORs were minimal, with age and stress exposure playing no role in these differences.

In summary, this study provides compelling evidence for age-dependent effects of stress on DYN/KOR system function. Given that the DYN/KOR system has been suggested to be a potential pharmacological target for stress-related disorders, such as anxiety and addiction (Chavkin and Koob, 2016; Tejeda et al., 2012), the present study suggests that work examining such potential pharmacological targets should carefully consider age and timing of stressor exposure.

Acknowledgements

Research reported in this publication was supported by the National Institute on Alcohol Abuse and Alcoholism of the National Institutes of Health under Award Numbers P50AA017823 and R03AA024890.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ynstr.2018.09.003.

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