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Effects of Antidepressants on Behavioral Assessment in Adolescent Rats

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Objective: To assess the usefulness of the forced swim test (FST) in juvenile rats and to determine the efficacy of two antidepressants on the behavioral changes of adult and juvenile rats.

Design: Animal interventional study.

Setting: Department of Pharmacology, Creighton University, Omaha, USA.

Method: Adult and 28 days old rats received saline, Imipramine or Fluoxetine three times during the 24 hours between 15 minutes pre-swim and the 5 minutes swim test. Immobility, swimming and climbing behaviors were measured. Locomotor activity was assessed for 28 days old rats in an open field test.

Result: Imipramine-treated juvenile rats had a decrease of immobility and increase climbing behavior, whereas Fluoxetine-treated juveniles had a decrease in immobility and increase in swimming behavior, both in a dose dependent manner.

Conclusion: The present study clearly showed that the antidepressant effects of Imipramine and Fluoxetine can be applied to juvenile rats in the forced swim test; it could be validated as practical model for screening of the antidepressant effects.

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The forced swim animal model is a common behavioral test for assessing depression in rodents and testing the efficiency of anti-depressants drugs. As originally developed by Porsolt in 1977, a rat is forced to swim for 15 minutes in a narrow cylinder from which there is no escape[1,2]. After an initial struggle the rat becomes immobile, that is, it floats while making only movements necessary to keep its head above the water. Administration of clinically effective antidepressant drugs prior to a second 5 minutes test swim session decreases the time an animal is immobile

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compared to saline treated animals, and thus the decrease in immobility time is used as an index of antidepressant activity. As a screen for drugs with antidepressant activity, the forced-swim test is selective for drugs which are clinically effective, and does not respond to most other classes of psychotropic medications[3]. False positive effects do occur with stimulants due to the generalized increase in activity, which is controlled for by use of an additional test for motor activity. A potential limitation of the forced-swim test is the short duration of drug treatment required to get an antidepressant effect. In rats 3 doses over 24 hours, and in mice a single dose, are sufficient to reduce immobility time, whereas the clinical elevation of mood may take several weeks of treatment. In addition, the interpretation of immobility as "behavioral despair" is controversial[3-5]. Thus, caution should be used in classifying the forced-swim test as a model of depression.

Despite some limitations, the forced-swim test is currently the most widely used preclinical *in vivo* test of antidepressant efficacy[6,7]. In the modified forced-swim test, increased water depth prevents rats from contacting the bottom of the tank[8,9]. A remarkable characteristic of the modified forced-swim test is that drugs which affect serotonin decrease immobility and increase swimming behavior, whereas drugs which affect norepinephrine also decrease immobility but increase climbing behavior.

Pediatric depression is a significant health issue, with a prevalence of 2.5% in children and 5-8% in adolescents[10,11]. By the end of adolescence, 20-25% of the pediatric population will have had an episode of major depressive disorder, with deleterious effects on social development, personality and the risk of self harm[11-14]. The presentation of major depressive disorder in the pediatric population, although similar to symptoms in adults, differs in that irritability, anger and anxiety are often present[15-17].

The developmental period is short in rats, they are born immature compared to humans, open their eyes at day 12-14, are weaned at day 21, and adolescence is generally defined as day 28 through day 42[18]. Although the FST model developed in adult animals can serve as starting point, it must be adapted and validated in juvenile animals due to many differences between juvenile and adult animals.

The aim of this study is to assess the usefulness of the forced swim test (FST) in juvenile rats and to determine the efficacy of two antidepressants on the behavioral changes of adult and juvenile rats.

METHOD

Animals

Juvenile and adult male Sprague-Dawley rats (Sasco/Charles River, Wilmington, MA) were used in these studies. Juvenile rats weighed 82 ± 10 gm on day 27 at the time of the swim pre-test, while adult male rats weighed 240 ± 15 gm. All animals were maintained on a 12-hour light: dark cycle (lights on at 7:00 am) in a temperature-controlled (22°C) room. Animals had free access to food and water, and were handled daily for at least 5 adaptation days before behavioral procedures. All experimental procedures were carried out in accordance with protocols approved by Creighton University, Omaha, USA Institutional Animal Care and Use Committee.

Forced-swim Test

The procedure used was based on that described by Porsolt[2] and modified to increase sensitivity by increasing water depth[8,9]. Behavioral studies were carried out in the afternoon (12:00 -17:00) under low illumination. Rats were placed individually in Plexiglas cylinders (Midwest Plastics, Inc., Omaha, NE) 46 cm in height with a 21 cm internal diameter that were filled with water (25ºC) to a depth of 30 cm. This depth was sufficient to keep adult rats from supporting themselves by placing their paws or tails on the base of the cylinder. Water was changed between each swim session to prevent possible effects of an alarm substance released by rats during the swim session[19]. There were two swim sessions. The first was 15 minutes pre-test swim and 24 hours later a second 5 minutes swim test. The pre-test facilitates the development of immobility during the test session and increases the sensitivity for detecting antidepressant behavioral effects[4]. The 5 minutes swim test was used for analysis of behavior. Rats received saline, Imipramine (1, 5, 10 or 20 mg/kg) or Fluoxetine (1, 5 or 10 mg/kg) by Intraperitoneal injection three times following the initial 15 minutes pre-test swim, at 23.5, 7 minutes and 1 hour prior to the 5 minutes swim test. Adult and juvenile saline groups were 12, and for each drug concentration, the treatment group size was 8.

Open-Field Test

Locomotor activity was assessed for day 28 rats in an open field test. Drug treatments used the same schedule as for the forced-swim test, three intraperitoneal injections at 23.5, 7 minutes and 1 hour before the open field test. Locomotor activity was compared between saline treated rats and rats receiving either Imipramine (20 mg/kg) or Fluoxetine (10 mg/kg), the highest doses of antidepressants used in the forced-swim test. The open-field test chamber consisted of a plastic bin (56 x 38 x 30 cm) with nine 12.7 x 7.6 cm square grids clearly drawn on the surface. An observer counted the number of line crosses during the 10 minutes test, recording the total line crosses at the end of each minute interval. Total grid line crossings during the 10 minutes were compared between treatment groups.

Behavioral and Statistical Analyses

The 5 minutes swim test session was videotaped, and each session was stored as a video file. A time-sampling technique was used, whereby the predominant behavior in each 5 seconds interval of the 5 minutes test swim was scored[9]. Immobility was assigned when the rat demonstrated floating behavior with no additional activity other than that required to keep its head above the water. Swimming behavior was defined as movement on the water surface throughout the swim chamber, with the rat in a horizontal position; climbing behavior was defined as upward-directed vigorous thrashing movements with the forepaws, usually along the side of the swim chamber. The three observers independently scored three adult and three day 28 test sessions. Each video was scored two or three times. The kappa coefficient indicates the strength of agreement, between scoring for an individual rater or among several raters, as follows: 0-0.20 poor; 0.21-0.40 fair; 0.41-0.60 moderate; 0.61-0.80 good; and 0.81-1.00 very good[20].

Behavioral scoring for drug treatment effects on forced-swim test behaviors was done by one rater who was blind to the experimental conditions. The rater was trained to score behaviors separately for adult and day 28 rats just prior to data collection by scoring reference videos for

each age until intra-rater reliability > 85% was achieved. In the forced-swim test the two test drugs were analyzed individually, using immobility, swimming and climbing as endpoints, whereas age (day 28 versus adult) and drug dose (0, 1, 5 and 10 mg) for Fluoxetine, or (0, 1, 5, 10 and 20 mg) for Imipramine, were the predictors. Two approaches were used: (1) the three endpoints were analyzed together as a random vector using 2-Way Multivariate ANOVA (MANOVA); (2) each endpoint was analyzed individually using a 2-Way ANOVA, followed by Bonferroni post tests. The significance level was set at $p<0.05$. Open-field data were analyzed by one-way ANOVA, followed by Dunnett's Multiple Comparison Test post test, with saline treatment as the control group.

RESULT

Reliability of Behavioral Analyses

Intra-rater reliability for each observer and inter-rater reliability among observers is presented in Table 1. Each rater rescored adult forced-swim tests with very good or good reliability, as assessed by kappa statistic coefficients. Percent agreement between repeat scoring for the composite of all behaviors and for each individual behavior ranged from 86 to 97% for adults and ranged from 79 to 93% for juveniles. Inter-rater reliability was very good for both adult and juvenile forced-swim test repeat scorings.

Table 1: Reliability of Behavioral Scoring in the Forced-Swim Test

				Individual Behaviors		
		Kappa (95% C.I.)	Behaviors Composite	Immobility %	Swimming Agreement	Climbing
Intra-rater reliability						
Adult						
	Rater 1	0.87** (0.76, 0.98)	90 ± 5	91 ± 3	88 ± 8	91 ± 5
	Rater 2	0.97** (0.85, 1.00)	94 ± 4	90 ± 8	95 ± 5	97 ± 3
	Rater 3	0.78 * (0.72, 0.85)	90 ± 4	86 ± 5	87 ± 9	96 ± 1
Juvenile						
	Rater 1	0.68 * (0.56, 0.80)	88 ± 2	86 ± 4	87 ± 7	91 ± 6
	Rater 2	0.77 * (0.65, 0.89)	89 ± 5	90 ± 8	83 ± 8	93 ± 8
	Rater 3	0.62 * (0.55, 0.68)	83 ± 7	92 ± 5	81 ± 14	79 ± 11
Inter-rater reliability						
Adult		0.82** (0.75, 0.88)	91 ± 3	89 ± 2	90 ± 4	95 ± 4
Juvenile		0.85** (0.78, 0.92)	88 ± 3	90 ± 3	85 ± 6	89 ± 9

*Good strength of agreement; **Very good strength of agreement

Antidepressant Efficacy in Adult and Juvenile Forced-swim Test

Fluoxetine: The effects of Fluoxetine are illustrated in Figure 1. In both adult and juvenile rats, Fluoxetine produced a dose-dependent decrease in immobility and an increase in swimming, with no effect on climbing. Analysis of the three behaviors together using a 2-way multivariate

ANOVA demonstrated a significant interaction between age and drug dose ($p<0.05$). Each behavior was analyzed individually by a 2-way ANOVA.

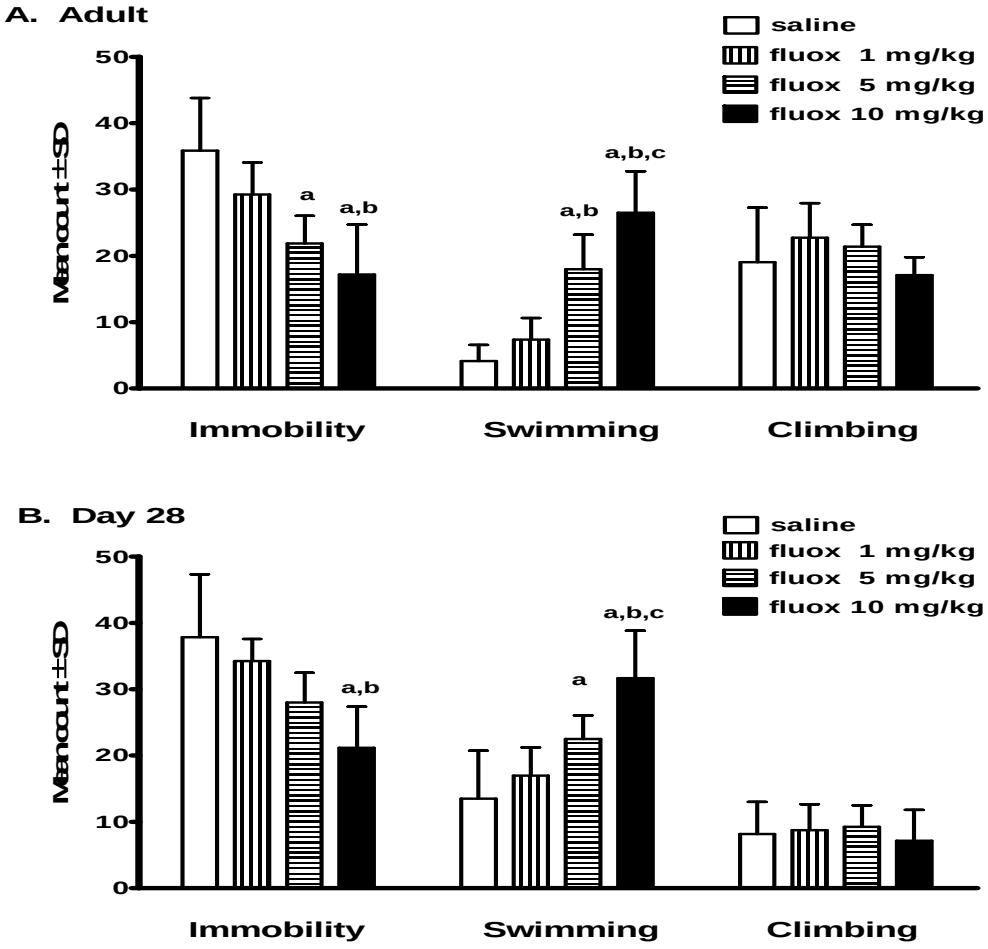

Figure 1: Mean Counts of Immobility, Swimming and Climbing in the Modified Forced Swim Test of Adult and Juvenile Rats Treated with Fluoxetine

The effect of Fluoxetine on immobility showed that age ($p=0.0238$) and drug dose ($p=0.0001$) were significant, but that their interaction was not significant ($p=0.1124$). The lack of interaction between age and drug dose indicates that adult and juvenile rats respond to different doses in the same way. Total immobility time for adults was significantly lower than for juveniles.

The effect of Fluoxetine on swimming behavior was very significant for both age ($p<0.0001$) and drug dose ($p<0.0001$), but their interaction was not significant ($p=0.1632$). Total swim time for adults was significantly lower than for juveniles. Adult rats had significantly higher levels of climbing behavior than juveniles ($p<0.0001$), but different doses of Fluoxetine ($p=0.1227$) and the interaction of age and dose ($p=0.7130$) were not significantly different.

Imipramine: The effects of Imipramine are illustrated in Figure 2. In both adult and juvenile rats, Imipramine produced a dose-dependent decrease in immobility and an increase in climbing,

with no effect on swimming. Analysis of the three behaviors together using a 2-way multivariate ANOVA demonstrated a significant interaction between age and drug dose (*p<0.05*). Each behavior was analyzed individually by a 2-way ANOVA.

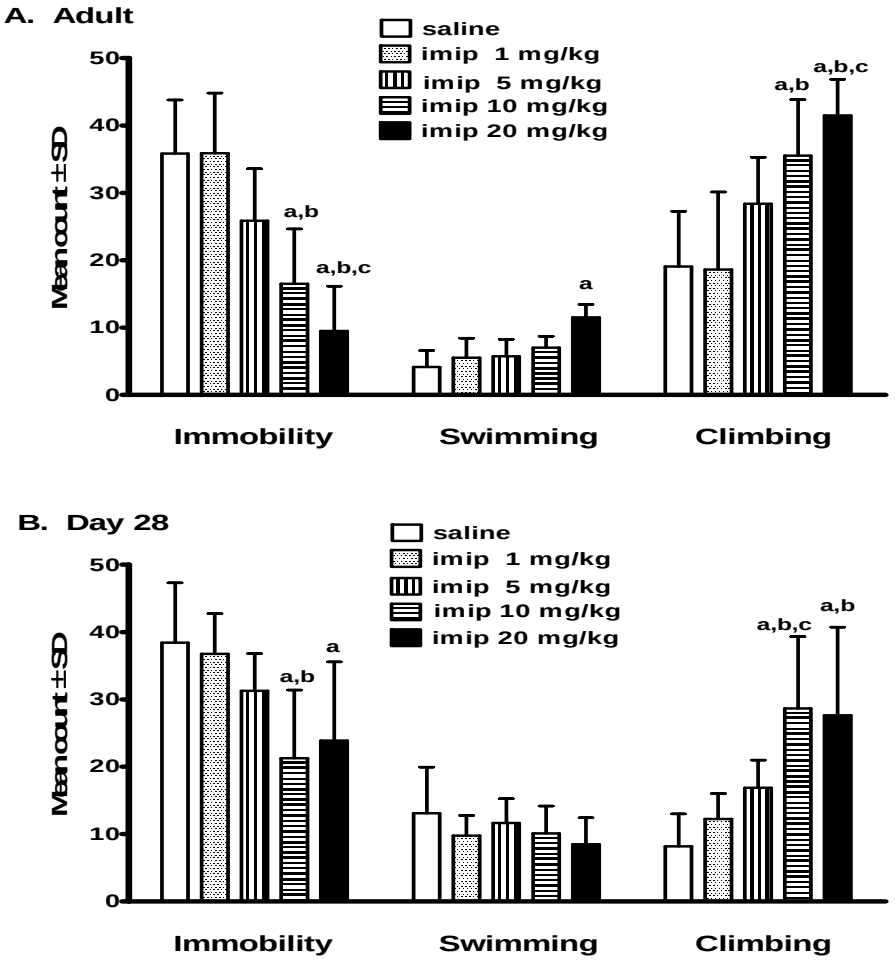

Figure 2: Mean Counts of Immobility, Swimming and Climbing in the Modified Forced Swim Test of Adult and Juvenile Rats Treated with Imipramine

The effect of Imipramine on immobility indicated that age (*p=0.015*) and drug dose (*p=0.001*) were significant, but their interaction was not significant (*p=0.288*). The lack of interaction between age and drug dose indicates that adult and juvenile rats respond to different doses in the same way. Total immobility time for adults was significantly lower than for juveniles.

For swimming behavior there was a significant interaction between drug dose and age (*p=0.0029*). There was no significant dose-dependent effect of Imipramine for swimming behavior in juvenile rats (*p=0.1568*), while for adults, it was significant (*p=0.0122*).

There was a dose dependent increase in climbing behavior for adult and juvenile rats. Imipramine showed both significant age (*p<0.0001*) and dose (*p<0.0001*) effects, but there was no significant interaction (*p=0.6102*). Total climbing time is significantly higher for adults than

for juveniles.

Locomotor Activity

The effects of Fluoxetine and Imipramine on locomotor activity of the juvenile rats are shown in Figure 3. Fluoxetine at 10 mg/kg had no significant effect on locomotor activity as compared to saline treatment while Imipramine at 20 mg/kg dose significantly decreased activity by about 50% ($p<0.05$).

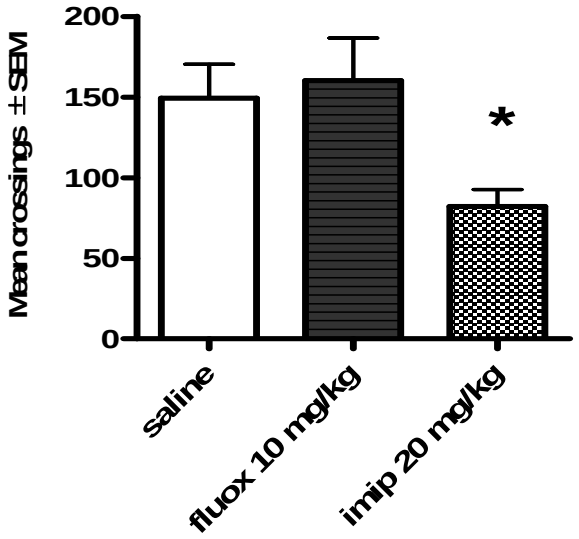

Figure 3: Effects of Fluoxetine and Imipramine on Locomotor Activates of Juvenile Rats in the Open-field Test

DISCUSSION

Comparison of Adult and Juvenile rats in the Forced-swim Test

We can cautiously assume that juvenile rats demonstrate the same pattern of behavior, exhibiting immobility, swimming and climbing behavior as seen with adult rats. Moreover, antidepressant drugs decrease immobility, demonstrating that reduced immobility is a good index for antidepressant efficacy in juvenile rats. The SSRI Fluoxetine is effective in both adult and juvenile rats. Fluoxetine treatment decreased immobility and increased swimming behavior in adults and juvenile rats. The TCA antidepressant Imipramine is also effective at this age. Imipramine treatment decreased immobility and increased climbing behavior in both adult and juvenile rats. Response to treatment with both drugs was dose dependent and indicated that there was no difference in dose-response between adult and juvenile rats.

Small differences exist in the amount of time spent in each behavior between juvenile and adult rats. Juvenile rats showed an increased immobility and swimming and decreased climbing behavior, compared to adults. In addition, our data showed that for each drug and each behavior, age was a significant factor, indicating differences in behavioral scores between the juvenile and adult animals. These differences may be attributed to several biological factors such as, variation

in metabolic enzymes activities, pharmacokinetics and pharmacodynamic factors during development differences in body fat and muscle composition, total body energy reserves, hormonal changes or the differential maturation of the noradrenergic versus serotonergic neurotransmitter systems[21,22].

Methodological considerations may also contribute to differences in behavioral scores between the juvenile and adult animals. Experimentally, an issue of potential concern is the 30 cm water depth we used for the FST, which would be relatively deeper for juvenile rats compared to adults, and the surface area of the tank used, both known to affect immobility and swimming[9,23,24]. In this study, the same tank size was used for both adult and juvenile rats. The ratio of surface area to body size is increased for juvenile rats, which may help explain the increased swimming behavior and decreased climbing at this age, as compared to adult behavior. Nonetheless, using the same tank size for juveniles and adults did not affect the ability to detect antidepressant-like effects and different behavioral profiles for the SSRI and TCA treatments.

Dose-response curves for each behavior and for both Fluoxetine and Imipramine were similar for juvenile and adult rats, indicating a comparable sensitivity to antidepressants of both noradrenergic and serotonergic systems. In addition, it indicates that potential differences in metabolism between juveniles and adults did not significantly affect the forced-swim test.

The effect of Imipramine on active behaviors in the forced-swim test was primarily to increase climbing behavior, indicative of a norepinephrine-mediated antidepressant effect[5]. Combinations of SSRI and norepinephrine selective drugs, as well as, the dual inhibitor Venlafaxine can lead to a simultaneous increase of both swimming and climbing[25,26]. Imipramine is rapidly metabolized to desipramine, which is highly selective for the norepinephrine transporter in the rat[27,28]. However, in adult rats, Imipramine at the highest dose tested of 20 mg/kg did have a small additional effect to increase swimming behavior, indicating an effect on serotonin levels. The effect of Imipramine on swimming was not observed with juveniles. Differences between juvenile and adult rats may be due to more rapid metabolism of Imipramine to desipramine in juveniles, or possibly to the selective accumulation of desmethyldesipramine (major metabolite of desipramine) in the adult rats. Desmethyldesipramine is more selective for inhibition of serotonin transporters and differences in its accumulation have been demonstrated between adult and juvenile rats[26,29].

Stimulant drugs, such as amphetamine or cocaine, can decrease immobility in the forced-swim test[3,30,31]. This is a false positive result and is distinguished from the antidepressant-like reduction in immobility by assessing locomotor activity in the open field test. In the juvenile rats, the highest dose of Fluoxetine did not affect locomotor activity, while Imipramine significantly decreased locomotor activity at the highest dose. Previous studies have demonstrated a similar decrease in locomotor activity for adult rats in the open-field test[25,32,33]. Despite the decrease in locomotor activity, climbing behavior was increased and immobility behavior was decreased. Therefore, the increase in active behaviors and decrease in immobility observed with antidepressant drug treatments was not due to an increase in locomotor activity.

Application to Pediatric Depression

Currently Fluoxetine is the only antidepressant approved for childhood and adolescent

depression, although other SSRIs and some atypical antidepressants are effective and used off label[34,35]. The evidence of lack of efficacy of TCA antidepressants, such as Imipramine and others, as well as, the potential for serious cardiotoxicity and the overall side effect profile has limited the use of these drugs for pediatric depression and no drugs from this class have been included in recent clinical trials[36-38]. On the other hand, in adolescents over 12-13 years, overall antidepressant response was much better than children were, and adolescents responded to a broader range of drugs, including several SSRIs and atypical antidepressants like venlafaxine, nefazodone and mirtazepine. However, adolescent response was still lower than in adults. It has been hypothesized that the lack of efficacy for TCA antidepressants and the efficacy of SSRIs in pediatric depression may be due to differences in the maturation of serotonergic and noradrenergic neurotransmitter systems[23,35]. Serotonergic system reaches maturity much earlier than the noradrenergic system in both rats and primates[22].

Rather than showing a difference in TCA efficacy, our results demonstrate that by early adolescence the antidepressant-like response in the forced-swim test is sensitive to both the SSRI Fluoxetine, as evidenced by decreased immobility and increased swimming, and the TCA Imipramine, as evidenced by decreased immobility and increased climbing. The increased climbing in response to Imipramine is an indication that the antidepressant-like effect is mediated primarily through the noradrenergic system. Therefore, we conclude that by early adolescence the serotonin and the noradrenergic systems are sufficiently developed to mediate depressive-like behaviors (immobility) and antidepressant efficacy (active behaviors) in the rat. Although this finding may indicate that the adolescent rat model may not be ideal for determining antidepressant efficacy related to human pediatric depression, issues related to species differences in development and careful interpretation of the clinical data need to be taken into account.

Species exhibit different patterns of ontological development. For example, the rat is born at a less mature stage, more equivalent to the third trimester of human development. The rat also reaches reproductive capability late in adolescence, only after establishing independent living, having left the nest in the wild at about day 28[18]. This is different from the human condition where reproductive capability is reached early in adolescence and before independent living. Across mammalian species, there are similarities in the order of major landmarks in brain development, but there are also differences in the timing of these events between species[39]. Brain development in general and especially development of the noradrenergic system continue through the adolescent period and into young adulthood[18,22,40].

These results demonstrate that the clinical efficacy of antidepressants increases with age. During childhood and adolescence, both the serotonin and norepinephrine systems are present, although still immature. Since the serotonin system matures before the noradrenergic system, one interpretation is that antidepressant efficacy improves as the noradrenergic system matures. In addition, there is an increasing evidence that the interaction between noradrenergic and serotonin systems is important in antidepressant mechanisms[39,41,42]. In animal studies, this has recently been shown for adult mice using the tail suspension test, a behavioral antidepressant screening model related to the forced-swim test. Thus, the development of coordinated interactions between serotonin and norepinephrine systems may be more important in increasing efficacy of antidepressants in pediatric depression than the timing of maturation of serotonin versus noradrenergic systems. Reduction of noradrenergic function by drug depletion or by examination

of animals at early ages, with a less developed noradrenergic system, could be used to determine effects on antidepressant efficacy in the juvenile forced-swim model.

CONCLUSION

The present study demonstrates the usefulness of the forced-swim test in juvenile rats, and in early adolescence, rats exhibit a similar response to antidepressant drugs Imipramine and Fluoxetine as do adult rats. Our results suggest that in the day 28 rat the behavioral effects of Imipramine are mediated mainly through the noradrenergic system as indicated by the increase in climbing, but not swimming, behavior. It is suggested that the use of the newer selective norepinephrine reuptake inhibitors and especially dual reuptake inhibitors should be revisited in adolescent depression.

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Potential Conflicts of Interest: No

Competing Interest: None, **Sponsorship:** None

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Ethical approval: All experimental procedures were carried out in accordance with protocols approved by Creighton University, Omaha, USA Institutional Animal Care and Use Committee

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