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Submissive behavior in mice as a test for antidepressant drug activity

Ewa Malatynska [a,c,*], Robert Rapp [a], David Harrawood [b], Godfrey Tunnicliff [b]

[a] Department of Pharmacology & Toxicology, Indiana University School of Medicine, Evansville, IN, USA
[b] Biochemistry & Molecular Biology, Indiana University School of Medicine, Evansville, IN, USA
[c] Johnson & Johnson PRD L.L.C., P.O. Box 776, Welsh and McKean Rds., Spring House, PA 19477-0776, USA

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Abstract

Previously, with the administration of antidepressant drugs, it has been demonstrated that the rat model of clinical depression, known as the reduction of submissive behavior model (RSBM), has considerable validity. The present study is an attempt to extend the model to mice. Several antidepressant drugs as well as a number of non-antidepressant agents were administered to mice that had been identified as submissive in a behavioral testing situation. Imipramine, desipramine, amoxapine and fluoxetine, representing three different classes of antidepressant drugs, were each able to increase competitive behavior in submissive mice and to decrease the dominance level between dominant and submissive mice in the behavioral tests. The stimulant amphetamine also reduced submissive behavior while yohimbine (also a stimulant), and the antianxiety agent diazepam had no such effect. The neuroleptic drug thiothixen had antidepressant-like effect on submissive C57BL/6J mice behavior. We conclude that like the rat model of depression from which it was developed, the mouse model responds to various antidepressants as predicted and thus may serve as a potential model of clinical depression.

Keywords: Dominant–submissive relation; Depression; Mouse model; Antidepressant drugs

1. Introduction

Clinical depression, including unipolar and bipolar disorders, is a major health concern (Robins et al., 1987). Our understanding of the etiology behind these disorders is far from complete, though theories involving various neurotransmitter systems have been advocated (Schildkraut, 1965; Duman, 1999; Tunnicliff and Malatynska, 2003). Unfortunately a sizable proportion of sufferers are resistant to antidepressant drug treatment, showing that more effective medications are needed (Greden, 2002). Major tools in the armament of research scientists seeking potential antidepressant drugs for the eventual treatment of clinically depressed patients are animal models of depression. Several such models exist and they can assist in the identification of drugs that could be suitable for clinical trials. These models, moreover, can be of immense value in helping unravel the neurochemical events responsible for deficits in affect.

Some models of depression can be evoked by the administration of drugs, an example being the clonidine-induced reversal of dominance behavior (CRDM) in rats (Malatynska and Kostowski, 1984). Other models such as the forced swim test (Porsolt et al., 1977), chronic mild stress (CMS) (Willner et al., 1992a,b; Papp et al., 1996) or resident intruder test (Mitchell et al., 1991; Kudryavtseva et al., 1991) rely on environmental manipulations. Recently a novel rat model of depression has been described and convincing evidence presented as to its validity for studying affective disorders (Malatynska et al., 2002). This model is known as the reduction of submissive behavior model (RSBM). It is based on social behavior and it evolved from the CRDM. Yet it does not rely on clonidine to effectuate the depression-like symptoms. Instead, certain rats in a population can be shown to exhibit submissive behavior when paired to a dominant partner. Submissive or dominant behavior is identified when randomly paired rats compete for food in an apparatus first described by Malatynska and Kostowski (1984). A dominant–submissive relationship develops over a two-week period and lasts for at least five weeks (Malatynska et al., 2002). This model already has been used to demonstrate an antidepressant

* Corresponding author. Johnson & Johnson PRD L.L.C., P.O. Box 776, Welsh and McKean Rds., Spring House, PA 19477-0776, USA.
E-mail address: Emalatyn@PRDUS.jnj.com (E. Malatynska).

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action of a number of memory-enhancing drugs (Knapp et al., 2002).

Mice can offer many advantages over rats in behavioral studies, the greatest one being the vast database of genetic information readily accessible on this species. For instance, a large number of distinct mouse strains have been developed over many decades. So-called knockout strains are additional sources of mice available with specific genetic modifications. Moreover, the use of ethyl-nitrosourea mutagenesis has led to the large-scale production of mouse mutants. The identification of genetic elements in mice associated with depression-like behavior can be tested for homology in patients that could lead to an understanding of the fundamental defects underlying clinical depression. This study describes results of an attempt to adapt rat RSBM to mice. We selected the C57BL/J6 mouse strain because (1) depressive-like behavior in this strain was demonstrated in a social test (Kudryavtseva et al., 1991), and (2) this strain is often used as a background strain to produce mutant mice.

2. Materials and methods

2.1. Subjects

The animals we used were C57BL/6J adult male mice purchased from the Jackson Laboratory (Bar Harbor, Maine). These studies were carried out using a protocol approved by the IUACUC at Indiana University and in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the United States National Institutes of Health. Mice had limited access to food. Typically, during each 24 h period, mice were food-deprived for 8 h. The exceptions were experiments with fluoxetine. During these experiments mice were fed only for 5 h a day following the testing session. From Friday midday after testing, animals had free access to food. This continued until Sunday evening when again they were deprived of food. All mice in the study showed satisfactory weight gain. The average mouse weight was 16.2 +/−2.7 on the beginning of the study and 21.8 +/−2.4 (mean +/−SD) on the end of the study. There was not statistically significant difference between dominant and submissive mice weight.

2.2. Drugs

Drugs were purchased from SigmaAldrich (St. Louis, MO), except fluoxetine, which was obtained from Tocris, Inc (Ellisville, MO). Other than diazepam, all drugs were dissolved in saline. Diazepam (5 mg) was mixed with 0.5 ml of 1 mM HCl and left overnight on a shaker. The solution was then diluted with 4.5 ml saline. Drugs were injected intraperitoneally once a day for 21 days. The dose of the antidepressants, fluoxetine ($n=4$), desipramine ($n=7$), imipramine ($n=7$), amitriptyline ($n=6$), and amoxapine ($n=8$), was 10 mg/kg. However, some animals received amoxapine at 1 mg/kg ($n=8$), and other animals received a 5 mg/kg dose of fluoxetine ($n=4$). Other drugs – thiothixene ($n=6$), yohimbine ($n=8$), amphetamine ($n=8$), and diazepam ($n=6$) – were administered at a dose of 1 mg/kg. In addition, some animals were given diazepam at a dose of 2 mg/kg ($n=6$). Doses of all drugs were determined on the basis of their activity in the rat RSBM (Malatynska et al., 2002), as well as in other models used to test in vivo activity of these drugs. Generally, we tried to avoid sedative effects, if expected, by using lower non-sedating doses. All drugs were dosed as free base equivalents.

2.3. RSBM procedure

Details of the equipment and the procedure in the rat model have been published (Malatynska et al., 2002; Pinhasov et al., 2005). For the present study the apparatus was constructed at Indiana University and was a scaled down version of the apparatus that was used for rats. The equipment is made of Plexiglas and consists of two identical chambers ($12\times8.5\times7$ cm) joined by a $2.5\times2.5\times27$ cm passage. In the center of the passage is a hole cut on the floor. A beaker filled with sweetened milk is placed in the hole before the start of the behavioral testing procedure. Before any testing, mice were adapted to a reverse day/night cycle for one week (dark from 6:00 A.M. to 6:00 P.M. and light from 6:00 P.M. to 6:00 A.M.). They were then randomly assigned to pairs. These pairs were brought together only once a day during a testing period. Otherwise the mice from pairs were separated to different home cages. The animals were housed in groups of four per home cage. On weekdays behavioral testing was conducted during the morning for a 5 min period each day.

The time spent on the feeder by each animal was recorded. For most of the test duration, the apparatus allowed only one mouse to feed at a time, though during each interval both animals could have consumed milk. Dominance was assigned to the animal with the higher score during the second week of testing (selection week), if there was a significant difference between the average daily drinking scores of both animals. On the third week, the drug treatments (once a day) began and were conducted everyday, including weekends. The individuals observing the behavior were unaware of the particular treatment each mouse had received. The dynamics of the experimental procedure and the number of animals entering experiment every second week is shown in the Table 1.

The significance of the differences between time spent on the feeder by dominant and submissive mice were determined by ANOVA using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA) followed by a two-tailed t-test ($P>0.05$). Comparisons were made between dominant and submissive animals on the initial week (before treatment) and each treatment week. Any loss of significant difference between animals indicated a change in their relationship

Table 1
Timetable for basic experimental unit

Procedure	Time	No. of animals	No. of animals selected	No. pairs with D/S relation
Habituation	5 days	32		
Selection	5 days	32	10–14	5–7
Drug dosing	3–6 weeks	10–14		5–7

(intra-pair control). Within a group of submissive animals the difference between initial (before treatment) week and subsequent weeks after treatment was also analyzed. A significant difference indicated a change in the attitude of the animal (internal control). Dominance level is a value that we use to measure social relation between two mice competing for food. This value is sensitive to the behavior of both animals in a pair that changes under the influence of a drug or other conditions. Dominance level = FTD − FST where FTD is the feeder time of dominant mice and FTS is the feeder time of submissive mice. In order to eliminate the influence of other conditions, the dominance level in an animal pair where the submissive animal was treated with drug was compared to the dominance level in an animal pair where the submissive animal was treated with vehicle (external control). The normalization was necessary to account for individual differences in the initial score level in pairs under different treatment. The normalization was conducted according to the formula $FT_{AVG\ n\ week}*100/FT_{AVG\ 2\ week}$, where FT = time spent at the feeder by an animal, AVG=average. The statistical significance of the difference in dominance level between the control group (pairs of mice were both dominant and submissive mice were treated with vehicle) and the treatment group (submissive mice was treated with drug and dominant mice with vehicle) was determined by ANOVA, followed by a *t*-test.

3. Results

3.1. Stability of the dominant–submissive relations in the RSBM

In a similar manner to rats, mice did not develop any dominant–submissive relationship during the first week of study (habituation week; data not shown). During this time, each mouse familiarizes himself with the apparatus and with his partner. Dominant–submissive relationships (DSR) were established over a second-week period and are shown as a statistically significant difference between dominant and submissive animals (Figs. 1–3). These relationships were unchanged in pairs of mice where both animals were treated with saline over the following 3 weeks (longest time studied). The data are presented on Fig. 1 A and B. The statistically significant difference between pairs of mice was maintained throughout the study. The persistence of formed dominant–submissive relationships is also shown by lack of a statistical difference in submissive mice scores (Fig. 1A) and in dominance levels (Fig. 1B) between initial week (before treatment) and weeks after treatment. However, mouse performance varied from week to week and dominance level has a tendency to decrease in vehicle treated animal pairs. This is why it is important to set the vehicle-injected group as an external control.

3.2. Effects of antidepressant drugs in the RSBM

Fluoxetine was ineffective at the lower dose (5 mg/kg, Fig. 2A). At this dose fluoxetine had a tendency to increase dominance level as compared with pairs of mice where submissive animals were injected with saline (Fig. 4A, B). This effect was statistically significant ($P<0.05$, two tail *t*-test) after the second week of injections. This increase was reduced after the third week of treatment (Fig. 4C). At the higher dose (10 mg/kg), fluoxetine produced a gradual reduction in submissive behavior. The significant difference in feeder time between dominant and submissive mice was lost after two weeks of treatment (Fig. 2B). The significant difference in dominance level between the control group and the group of mice injected with the higher dose of fluoxetine was achieved after three weeks of treatment (Fig. 4C).

Both imipramine and desipramine increased the competitiveness of the submissive mice after the first and second week of injections. The significant difference in time spent at the feeder between dominant and submissive mice was lost after the first and third weeks of treatment with desipramine (Fig. 2C) and after the third week of treatment with imipramine (Fig. 2D). The effect of both antidepressants on dominance level was

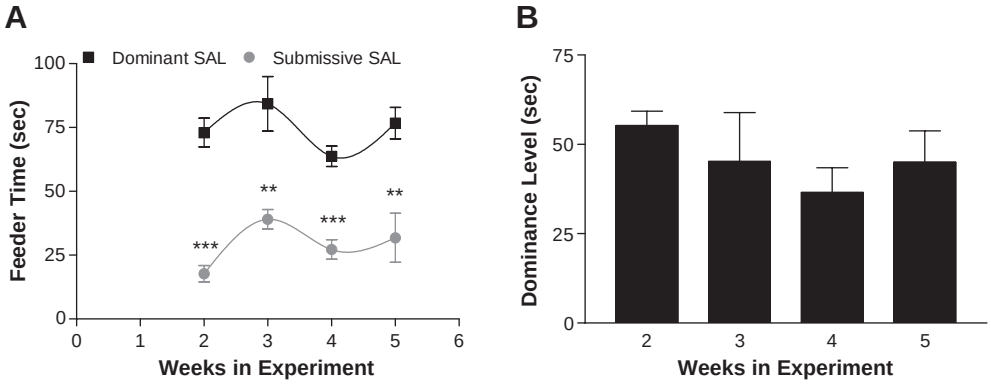

Fig. 1. Stability of dominant–submissive behavior in *C57/J6* strain of mice. A. Dominant–submissive relationships are shown in pairs of mice selected according to specific criteria (see Methods) after the second week of experimental sessions and its continuation during three weeks of treatment with vehicle, saline (SAL). Black squares (■) depict behavior of dominant mice, and gray circles (●) represent behavior of submissive mice. Statistically significant difference between dominant and submissive mice is marked as ** at $P<0.01$ and *** at $P<0.001$. B. Dominance level, the difference between dominant and submissive mice scores. There is no statistically significant difference between the second week (before treatment) and the following three weeks after treatment with vehicle, indicating no changes in dominance level.

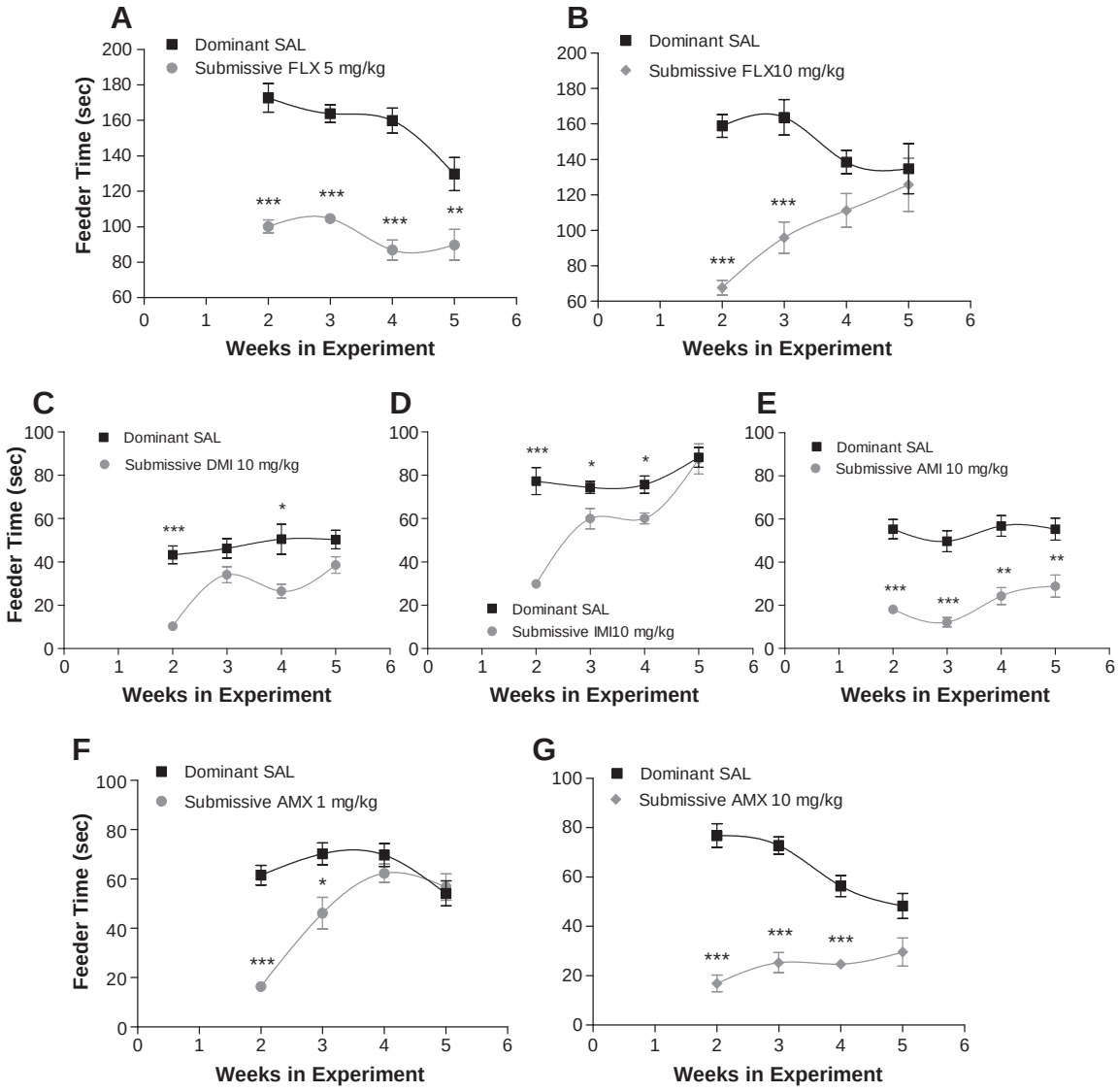

Fig. 2. Effect of antidepressant drugs in submissive mice. After selection, dominant animals received saline (SAL) while submissive animals received (A,B), serotonin reuptake inhibitor (SSRI), fluoxetine (A), 5 or (B), 10 mg/kg; , (C,D,E) tricyclic antidepressants: (C), desipramine (DMI), (D), imipramine (IMI) or (E), amitriptyline (AMI) 10 mg/kg; (F,G), heterocyclic antidepressant, amoxapine (AMX) (F), 1 or (G), 10 mg/kg. Statistically significant difference between dominant and submissive mice is marked as * at $P<0.05$, ** at $P<0.01$ and *** at $P<0.001$.

significantly different from control after the third week of administration (Fig. 4C). On the other hand, submissive mice injected with amitriptyline maintained significantly shorter time at the feeder compared to their dominant partners throughout the study (Fig. 2E). In fact, the antidepressant had a tendency to increase dominance level in the first and second week of the study (Fig. 4A and B). This tendency faded after the third week of treatment (Fig. 4C).

Amoxapine at 1 mg/kg produced a distinct reduction in submissive behavior. The significant difference in time spend on the feeder between dominant and submissive mice was lost after two weeks of treatment and persisted after the third week of treatment (Fig. 2F), whereas at the higher dose (10 mg/kg), this difference ceased to be significant after the third week of treatment (Fig. 2G). At the lower dose, amoxapine reversed dominance in pairs of mice as measured by dominance level after the second and third week of administration compared to saline injected control pair of mice (Fig. 4B and C). At the higher dose of 10 mg/kg this effect was significant after third week of treatment (Fig. 4C).

3.3. Effects of non-antidepressant drugs in the RSBM

The stimulant yohimbine at 1 mg/kg increased the competitiveness of submissive mice. The significant difference in time spent on the feeder between dominant and submissive mice was lost after two weeks of treatment and persisted after the third week of treatment (Fig. 3A). Yohimbine had a tendency to decrease dominance level, but this effect did not become significantly different from the control group with the duration of treatment (Fig. 4). The stimulant amphetamine also increased competitiveness of submissive mice. This is illustrated by the loss of significant difference in time spent at the feeder by dominant and submissive mice that occurred after the

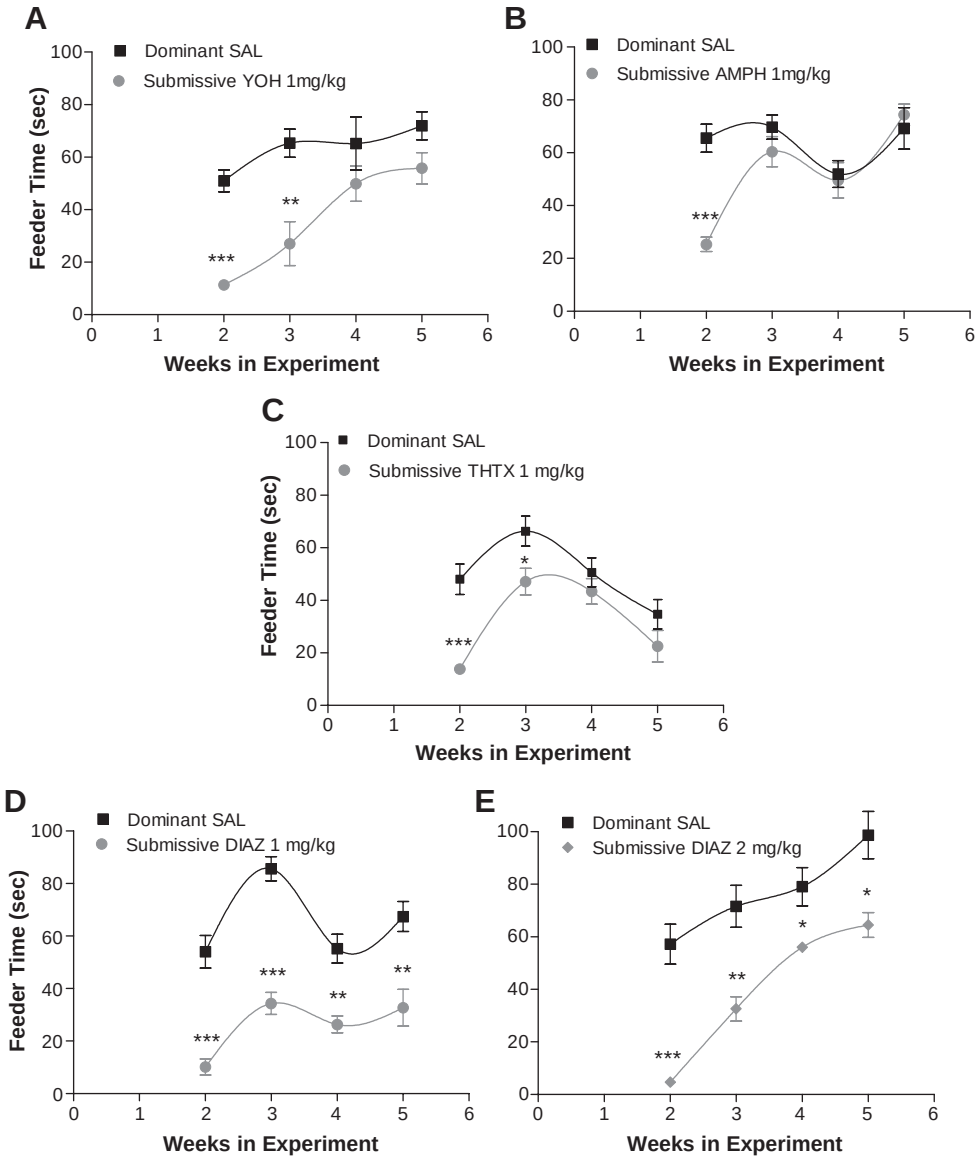

Fig. 3. Effect of non-antidepressant drugs in submissive mice. After selection, starting on the third week of experiments, dominant animals were injected with saline (SAL) while submissive animals were injected with (A), yohimbine (YOH) and (B), amphetamine (AMPH), 1 mg/kg, (C), thiothixene (THTX) 1 mg/kg, (D,E), diazepam (DIAZ) (D), 1 or (E), 2 mg/kg. Statistically significant difference between dominant and submissive mice is marked as * at $P<0.05$, ** at $P<0.01$ and *** at $P<0.001$.

first week of treatment and persisted for the following two weeks. Amphetamine decreased dominance level after the first, second and third week of injections (Fig. 4A, B and C).

The antipsychotic drug thiothixene (1 mg/kg) slightly increased competitiveness of submissive mice in the first week of treatment. This effect was short lasting, however, and the performance of the submissive mice returned to baseline values with the continuation of treatment (Fig. 3C). Unexpectedly, saline-treated dominant mice evidently also reduced their time spent on the feeder, but this reduction resulted in a reduced dominance level that was significantly different from control mice dominance level after second and third week of treatment (Fig. 4A, B and C).

The antianxiety drug diazepam, when administered at 1 mg/kg or 2 mg/kg, had no significant influence on submissive mice behavior (Fig. 1D and E). At the 2 mg/kg dose, diazepam had a tendency to reduce competitiveness of submissive mice. However, the statistically significant difference in feeder time of dominant and submissive mice was maintained for all three weeks of treatment (Fig. 3E), and the dominance level in the pairs of mice where submissive was treated with diazepam was not significantly different from dominance level in control pairs (Fig. 4).

4. Discussion

We have previously reported that a rat model of depression, that we called the Reduction of Submissive Behavior model, was sensitive to antidepressant treatment in a manner consistent with it being a valid model of clinical depression (Malatynska et al., 2002). In that model, submissive rats represent subjects with a clinical affective disorder. Indeed, others have presented

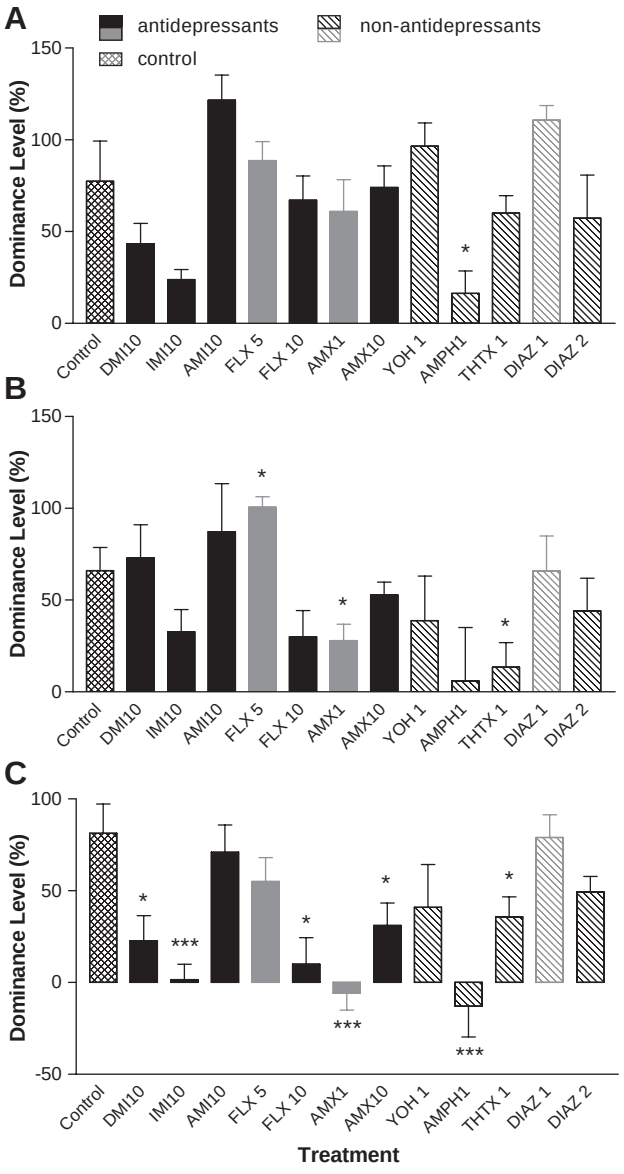

Fig. 4. Dominance level in the RSBM after treatment of submissive mice with either vehicle control (crossed bar), antidepressant (solid bars), or non-antidepressant drugs (hatched bars) for (A) one (B) two, and (C) three weeks. Light gray color for solid and hatched bars indicate lower dose for a given drug. Abbreviations are the same as in the legend for Figs. 2 and 3. Dominant animals were always treated with vehicle. DL=FTD FTS where DL is a dominance level, FTD is feeder time of dominant mice and FTS is feeder time of submissive mice. In this analysis feeder time (FT) was normalized to the initial week (second week) value according the formula $FT_{AVG5week}$*100/ $FT_{AVG2week.}$ Bars marked (*) have values significantly different from the values derived from corresponding week of control mice at $P<0.05$; and marked as (***) at $P<0.001$.

evidence that submissive animals are reminiscent of depressed patients (Blanchard et al., 1988, 1987; Gardner, 1982; Fonberg, 1974; Zagrodzka et al., 1985).

The present study tests the concept that submissive mice like rats can serve as a depression model. The results of our experiments have shown that dominant–submissive pairs of C57BL/6J mice can be established and that this relationship is stable for at least three weeks. Our observations support the work of other researchers (Kudryavtseva et al., 1991), as well as our experiments with the RSBM in rats (Malatynska et al., 2002). Like rats, submissive C57BL/6J mice become more competitive after treatment with tricyclic antidepressants, imipramine and desipramine, and selective serotonin reuptake inhibitors (SSRIs), fluoxetine. We have extended the range of drugs to include the tetracyclic antidepressant amoxapine and found that it also attenuated submissive mice behavior and decreased dominance levels. Amitriptyline, a tricyclic antidepressant, was the only antidepressant studied that did not reach significance after third week of administration, as previously shown in the rat RSBM (Malatynska et al., 1995). Amitriptyline at this dose had a tendency to worsen competitiveness of submissive mice after first week of dosing. This effect was reduced with the duration of treatment and the possibility exists that after a longer administration time this effect would be reversed. The same is true for fluoxetine at 5 mg/kg. Mice performed worst in the competition test up to the second week of dosing and this effect was reduced but not reversed after third week of administration. Fluoxetine at a higher dose (10 mg/kg) clearly increased competitiveness of submissive mice suggesting that amitriptyline, which was studied only at one dose, 10 mg/kg, may have antidepressant-like effect in RSBM at higher doses.

It is worth noticing that the reduction in submissive behavior after antidepressant drug administration, did not occur immediately but tended to develop gradually and to increase over the following few weeks. This parallels the delayed effectiveness of antidepressants observed in clinical trials and routinely seen in clinical practice.

Diazepam, an anxiolytic drug, was unable to influence the submissive behavior of mice when studied in two doses, 1 and 2 mg/kg. The diazepam effect on submissive mice was similar to its effect on submissive rats. However, in rats we have studied only one dose of diazepam, 1 mg/kg (Malatynska et al., 2002). Similar to diazepam, the psychostimulant yohimbine at 1 mg/kg did not significantly affect competitiveness of submissive mice as compared to saline-injected control mice.

We have previously shown that yohimbine decreased performance of dominant rats (Malatynska and Kostowski, 1984). However we did not study the effect of yohimbine in submissive rats. Perhaps, it would be different from that in mice. We found that another stimulant, amphetamine, acted in opposite way in submissive rats and mice. Treatment with amphetamine (1 mg/kg) led to an increase in drinking time in the submissive mice, producing a reduction of submissive behavior. Amphetamine was found to be inactive in the rat model of submissive behavior (Malatynska and Knapp, 2005). It is not obvious why treatment with these drugs produced a positive outcome in mice. It is of interest to note, though, that amphetamine and yohimbine can sometimes act as antidepressants in patients (Klerman, 1972; Sanacora et al., 2004). This is also true for the neuroleptic drug thiothixen (Goldstein and Brauzer, 1973; Gunderson, 1986; Simpson et al., 1972). Thiothixen reduced the dominance level in pairs of mice after two and tree weeks of administration as compare to water injected control pairs (Fig. 4). We have attempted to study the effect of another neuroleptic drug chloropromazine (data not

shown). However the strong sedative effect of this drug on C57BL/6J mice completely inhibited their activity including feeding and the study was not concluded.

Overall we have shown that antidepressant drugs from different classes including tricyclics, (imipramine, desipramine) tetracyclic, (amoxapine) and SSRI, (fluoxetine) reduced submissive mouse behavior while diazepam and yohimbine were devoid of this activity at the doses studied. Thus, mouse RSBM distinguished antidepressants with anxiolytic and psychostimulant activity. However, positive effects in the mouse RSBM were observed for drugs that are not classified as antidepressants including, thiothixen (neuroleptic) and amphetamine (psychostimulant). Generally, it is difficult to find negative controls for animal models of affective disorders due to the fact that drugs used to treat certain mental illnesses may have overlapping therapeutic activities. For example, amphetamine, yohimbine and thiothixen and even diazepam may have some antidepressant therapeutic activity (Klerman, 1972; Sanacora et al., 2004; Goldstein and Brauzer, 1973; Gunderson, 1986; Simpson et al., 1972; Petty et al., 1995). However, they are not used to treat depression due to their severe side effects e.g. abuse potential or extrapyramidal syndromes.

There are some noticeable differences between mouse scores among experiments that need to be addressed. First, pairs of mice where the submissive mice were treated with fluoxetine have higher scores for time spend on the feeder than in any other tested groups of paired animals. This difference results from a shorter feeding time 5 h (in experiments involving fluoxetine) versus 8 h (in experiments involving other drugs). Length of feeding time influences the time spent on the feeder by individual animals. For the detail discussion of this influence see (Malatynska and Knapp, 2005). Scoring of the animals by different observers may also result in distinct values of the feeding time. This prevents comparisons between groups observed by different experimenters. This is also the reason for normalization used for DL calculation (see Methods section). Such normalization enables comparisons between treatment groups. Observer-based differences among experiments can be also eliminated by automation of the RSBM. We have tested the automatic version of RSBM for rats (Pinhasov et al., in press) and plan to do such modification with the mouse model.

Second, the values of time spent on the feeder by dominant rats, treated with vehicle, who are partners for submissive rats, treated with the drug, may stay level, or may decrease or increase during experiment. In most cases this is a tendency only since there is no significant difference between initial week (selection week) and drug treatment weeks. To a certain extent these apparent phenomena are also observer-dependent. The decrease of the dominant partner score may be interpreted as an observer-dependent effect. Some observers pay more attention to the real drinking time than to animal presence in close proximity to the feeder. For most of the time it is possible to drink milk only for one animal so the feeder time of another animal from the pair is naturally reduced. This type of behavior of a partner animal is to a great extent reduced by the application of automatic scoring where the animal presence in the feeder zone is recorded instead of real drinking time (unpublished observation). However, there still is a small decrease or to a greater extent an increase in the feeder time recorded in untreated animal using automatic scoring. One possible interpretation is that the change in the behavior of treated animals has impact on the behavior of its partner. It is interesting to consider the three possible reactions of dominant partner. He either increases activity to overcome obstacle, stays unchanged or submits to the stronger expressed need of a partner. Furthermore, partners of antidepressant treated submissive animals either submit to the new situation or remain unchanged. While behavior of animals that are partners to submissive ones treated with other psychotropic drugs has a tendency to be parallel so in most cases it does not allow for the changes in a social status. For example such contrasting behavior of paired animals is observed in pairs where submissive mice were treated with amoxapine or diazepam (see Fig. 2G and E). Taken together we think that calculated DL values (Fig. 3) provides a valuable end point because it takes into account behavior of both animals in the pair that may change after drug treatment directly (treated animal) or indirectly (by the influence of a behavior of a treated animal). The social interactions by definition require at least two individuals and the change of social behavior by the drug must be also defined in such settings.

In conclusion, our new mouse model has a potential to be added to the available models of depression. We have established that several classes of antidepressants can reduce submissive behavior and that this effect tends to take a week or two before it is measurable. However, its predictive validity needs to be further studied with inclusion of newer antidepressants and other psychotropic drugs that could provide more firm negative control. Furthermore, the face validity of this model should be studied by assessing behavior of submissive mice in other models of depression. It would be also valuable to extend the drug testing time to 5 weeks and evaluate automatic version of this model. As we pointed out in the "Introduction" section, a mouse model will offer many advantages over the rat model from which it arose. These advantages include costs, as well as the large database of genetic information available on the laboratory mouse.

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