Full title: Mu-opioid receptor-dependent changes in social reward across adolescence in mice

Short title: Opioid-dependent changes in social reward in adolescence

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Abstract

In humans, adolescence is a time of dynamic behavioral changes, including a transient decrease in affect associated with being among family members. Here, we found that the reward value of interactions with siblings in adolescent male mice followed a similar course to that in humans: high in preadolescence, a decrease in mid-adolescence and a return to the initial level in late adolescence, as observed in the social conditioned place preference task. The observed change was specific to social interaction, as the rewarding effect of cocaine was actually increased during adolescence. Strikingly, treatment with a selective mu-opioid receptor antagonist, cyprodime, increased socially conditioned place preference in early adolescent mice, but not in older animals. Taken together, these data show similarities between mice and humans in developmental changes in sensitivity to the rewarding effects of interactions with familiar kin and demonstrate the involvement of endogenous opioid signaling in shaping adolescent social behavior.

Teaser

Adolescent mice experience changes in their affect towards kin in the same way as human teenagers, and this effect is dependent on opioid signaling.

INTRODUCTION

Adolescence is a time of rapid behavioral and neural changes, as well as the peak onset age for many mental disorders (*1*, *2*). It is postulated that the emergence of psychiatric symptoms during adolescence results from alterations in typical developmental processes (*3*). However, causal links between adolescent changes in brain maturation, behavior and pathophysiology have not been firmly established, partly because of the lack of proper animal models. It is thus of great importance to understand to what extent the behavioral development of model animals parallels the features of human adolescence (*4*).

One of the characteristic features of human adolescence is changes in social preferences. Whereas infants fully depend on parental care and display strong distress following separation from their mothers, adolescents show a decrease in the time spent with their family members along with

an increase in the time spent alone or with peers (*5*, *6*). This behavioral shift is accompanied by changes in emotions associated with relatives: early and late adolescents show positive emotions in the company of their family members, while mid-adolescents report negative emotional states (*5*). However, it is not known whether affective changes resembling those observed in humans also occur in animals. Therefore, the first goal of the present study was to assess changes in the social reward value of interactions with familiar kin across adolescence in mice.

Opioid receptors, and the mu-receptor in particular, play a key role in social behaviors (*7–9*). However, drugs acting on opioid receptors have opposite effects on social interactions in infants compared to adolescent or adult rodents (*7*). In particular, opioid agonists decrease maternal contact seeking in isolated rat pups (*10*), while opioid antagonists tend to increase this behavior (for review see (*11*)). In contrast, social play behavior in juvenile and adolescent rats is facilitated by opioid agonists and attenuated by opioid antagonists (*12–15*). These observations suggest that the contribution of mu-opioid signaling to social reward may change during juvenile and adolescent period. Examining this possibility was the second goal of our study. To this end, we investigated the effect of a mu-opioid receptor antagonist on social reward across adolescence.

RESULTS

Rewarding effects of interactions with siblings across adolescence

To examine the possible changes in the rewarding effects of social interactions with siblings during adolescence in mice, we used the social conditioned place preference (sCPP) test (**Fig. 1A,** (*16*)) with male mice at four ages: preadolescence (around postnatal day 33 [P33]) and early (P36), middle (P39) and late adolescence (P42) (for information about experimental groups, see **Tables S1** and **S2**). Animals were conditioned to associate one environmental context with group housing and another with social isolation and then were tested to determine context preference. Mice aged 33, 36 and 42 (but not 39) days at posttest showed a significant increase in the time spent in the social context from pretest to posttest (**Fig. 1B-E, Table S3**), indicating that interactions with siblings had lower reward value for mid-adolescent mice. The decrease in preference for the compartment associated with social contact in mid-adolescent mice was also clearly apparent in the social preference score (**Fig. 1F, Table S3**); this score showed that the rewarding effects of social interactions tended to be lower in mid-adolescent (P39) than in preadolescent (P33) mice and returned to the preadolescent level in late-adolescent (P42) mice. While mice tested at approximately P33 and P42 showed a mean score of 250-300 s, the mean preference was approximately 130 s at P39. The decrease in social score observed at P36 did not reach significance. Importantly, motor activity was not significantly affected by age (**Fig. 1G**); thus, it was not a confounding factor. Taken together, the results reveal a transient decrease in the rewarding effects of interactions with related individuals in adolescent mice.

Social contact

Next, we investigated if the change in social behavior in mid-adolescent mice was specific to the rewarding effects of social interactions or if contact seeking was also altered. To explore this possibility, we administered a test in which contact with another mouse is enabled through a transparent, perforated plexiglass wall (**Fig. 2A**) (*17*). The interaction partners were siblings reared in the same cage but isolated for one day before the test to match the conditions of the sCPP posttest. We observed no age-related changes in the time spent in the proximity to the partner (**Fig. 2B, Table S4**) or the distance between the focal mouse and the partner's compartment (**Fig. 2C**). Thus, the

decrease in the rewarding effects of interactions with related individuals in adolescent mice was not accompanied by a general decrease in social contact seeking.

Rewarding effects of cocaine

An alternative explanation for the observed decrease in the rewarding effects of social interactions could be a general impairment in associative learning. To test whether the decrease in the rewarding effects of social interaction reflected a stimulus-independent reduction in the expression of conditioned behaviors, the cocaine-induced CPP was assessed. We observed a significant increase in the time spent in the cocaine-associated context in early- and mid-adolescent (P36 and P39) mice but not preadolescent (P33) mice (**Fig. 3A-C, Table S5**). In the late-adolescent group, a trend toward an increase was observed, but it did not reach statistical significance (**Fig. 3D**). This effect was also apparent in the place preference score and index (**Fig. 3E-F**). Both measures showed higher rewarding effects of cocaine in early-adolescent (P36) than in late-adolescent (P42) mice. There was a significant effect of age on locomotor activity according to post hoc analysis; however, locomotor activity was not correlated with preference (**Fig. 3G**). These data show that no general impairment of associative learning occurred during adolescence. Our findings are consistent with previous reports of heightened sensitivity to drugs of abuse in adolescent animals (*18*).

The effect of cyprodime on the expression of socially conditioned place preference

Finally, we assessed whether the rewarding properties of interactions with siblings in adolescent mice are dependent on mu-opioid receptors. We applied a selective mu-opioid receptor antagonist, cyprodime (1 mg/kg, i.p.), at 1 h prior to posttest. In line with the previous result, in the control group, a U-shaped relationship between social reward and age was observed (**Fig. 4A-E**). Cyprodime increased the time spent in the social context of early-adolescent mice (P36), although the statistical significance of this increase was at the tendency level (p=0.05, **Fig. 4A**). Additionally, the drug had no effect in pre-, mid- and late-adolescent mice (**Fig. 4B-D**). Among the preadolescent mice (P33), 75% of the control group and 90% of the cyprodime group exhibited an increase in the time spent in the social context from pretest to posttest. These values in the early-adolescent (P36) group were 60% and 95%, respectively. For mid- and late-adolescent animals, the percentages of animals that exhibited an increase in the time spent in the social context after conditioning were similar in the saline and cyprodime groups (P39 saline: 73%, cyprodime: 85%; P42 saline: 80%, cyprodime: 75%). The social score followed a similar pattern; a significant effect of the drug was detected, which could be attributed mostly to the saline-drug difference at P36 (two-way ANOVA) (**Fig. 4E**). This effect was not explained by general changes in task performance, as locomotor activity was not altered by cyprodime in any age group (**Fig. 4F**). These data show that mu-opioid receptors play an important role in the regulation of social reward in early, but not in late adolescence.

DISCUSSION

We showed that the rewarding effects of interactions with familiar kin in male mice exhibit a transient decrease during adolescence (around P35-40). This decrease was specific to social reward, as no change in social contact seeking was observed, and the reward value of cocaine increased during this period. Importantly, rewarding effects of social interactions were age-dependently influenced by a mu-opioid receptor antagonist, cyprodime. Our results are markedly similar to

human data reported by Larson and Richards (1991), who observed that the affect associated with time spent with family members was more positive in 10-year-old and 16-year-old boys than in 12- to 14-year-olds (*5*). The age range of 11 to 16 years in male humans corresponds to P30-40 in male mice, and this period is considered "peripubertal" (*19*). This indicates that the phenomenon observed in our study, i.e., the temporary decrease in the reward value of interactions with familiar kin, is evolutionarily conserved. This finding may facilitate future research on the neuronal and physiological underpinnings of rebellious behaviors in adolescence.

Our results complement those of two previous studies that assessed the rewarding effects of social interactions using the sCPP paradigm at selected time points in adolescent mice (*20*, *21*). In contrast to our methods, both previous studies used animals that were familiar but were not specifically kept in sibling groups. As we have shown previously, regarding the reward value of social interactions, eight weeks of familiarization with nonrelated mice is not equivalent to being reared in the same cage as other mice before weaning, at least in females. Mice do not form an sCPP when conditioned as adults in groups coming from different litters and housed together since weaning (*16*). Moreover, both previous studies used a paradigm with only two days of conditioning, which produces different results than the paradigm with six conditioning sessions used in this study (*16*). Bearing these differences in mind, we note that Cann and collaborators (2020) reported that social contact is rewarding in mice tested on postnatal day 29 but not on postnatal day 38, which aligns with our results. Conversely, in the study by Nardou and collaborators (2019), a decrease in the rewarding effects of social interactions during adolescence was not observed in male mice (although it was observed in females). However, the previous results were not interpreted in terms of the possible decrease in the reward value of social interactions during adolescence.

In contrast to social reward, there were no apparent changes in social contact seeking (assessed with the partition test) during adolescence. This finding indicates that the changes in social interactions in adolescent mice are qualitative rather than quantitative in nature. We speculate that the amount of time spent with siblings may not change, but aggressive encounters may replace affiliative interactions. This interpretation is supported by earlier studies showing a profound decrease in passive social contact (*22*) and play behavior (*23*), along with an increase in fighting (*22*, *24*), in the second postnatal month of mice.

Strikingly, the cocaine-induced CPP appeared to follow the opposite pattern of the sCPP results. This result confirms that different neuronal processes underlie the rewarding effects of cocaine and social contact (*25*). Our results are also consistent with previous observations concerning the developmental changes in a cocaine-induced CPP in rats, a species in which the equivalent of P30-40 in mice is P42-55 (*19*). In rats, a greater cocaine-induced CPP in adolescents (P44) than in adults (P105) was reported by Brenhouse et al. (*26*). Notably, preadolescent rats (P27-37) showed similar levels of cocaine-induced CPP as adult animals (*26*, *27*), exhibiting the same inverted U-shaped relationship between cocaine reward and age as the one reported in the present study. Taken together, these results show that social and drug reward follow opposite developmental trajectories in the peri-adolescent period.

The effect of cyprodime, a selective mu-opioid receptor antagonist, on social reward is particularly interesting in the context of the brain opioid theory of social attachment (BOTSA (*28*)) and its later reformulations (*7*, *9*). The BOTSA predicts that low basal mu-opioid tone induces motivation for social contact seeking. This prediction is supported by studies on the effects of opioid drugs on separation-induced maternal calls in newborns (*11*). The levels of opioid peptides acting on mu receptors are very low in infants (*29*), which could explain why separation from the mother

causes extreme distress at this stage of life. Adolescents appear not to experience isolation-induced distress, as demonstrated by our present study and an earlier study in humans (*5*). Hence, opioid tone at this developmental stage may be exceptionally high. This interpretation is consistent with the mu-opioid receptor balance model (*9*), according to which hyperstimulation of mu-opioid receptors caused by intense/prolonged exposure to opioids saturates the reward system and results in indifference to social contact. Our results support this hypothesis, as the rewarding value of interactions with siblings in early adolescence was increased by administration a mu-opioid receptor antagonist. In line with this reasoning, D'Amato and collaborators observed higher expression of proenkephalin, a source of mu-opioid receptor agonist peptides, in the dorsal striatum of early-adolescent (P35) mice compared to adults (*30*). The dorsal striatum is one of the potential brain regions responsible for the differences in reward processing between adults and adolescents (*31*, *32*). Moreover, the involvement of the dorsal striatal mu-opioid system in social preference behaviors was suggested by a study in monogamous voles (*33*) in which administration of a mu-receptor ligand into the caudate putamen (rather than some areas of the nucleus accumbens) regulated partner preference. Future studies should investigate whether and how the mentioned age-dependent differences in the activation of striatal neurons are associated with the differences in the rewarding effects of social interaction.

METHODS

Animals

Experiments were performed with C57BL/6 male mice bred at the Maj Institute of Pharmacology animal facility. Mice were housed in a 12/12 h light-dark cycle (lights on at 7 AM CET/CEST) under controlled conditions: a temperature of 22 ± 2 °C and a humidity of 40-60%. After weaning, the mice were housed with all littermates of the same sex. Rodent chow and water were available ad libitum. Home and conditioning cages contained aspen nesting material and aspen gnawing blocks. Behavioral tests were conducted during the light phase under dim illumination (5-10 lux). sCPP and social interaction tests were video recorded with additional infrared LED illumination. The age and weight of mice in each experimental group are summarized in **Table S1**.

All behavioral procedures were approved by the II Local Bioethics Committee in Krakow (permit numbers 35/2019, 265/2019, 185/2020, 266/2020, 305/2020, 32/2021) and performed in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The reporting in the manuscript follows the ARRIVE guidelines.

Social conditioned place preference

The test was performed to assess the rewarding effects of housing with siblings and followed the procedure described previously (*16*). The test consisted of three phases: pretest, conditioning, and posttest (**Fig. 1A**).

The pretest and posttest phases were performed in a two-compartment cage, as in previously published papers (*16*, *21*, *25*, *34*, *35*). Each cage compartment contained a novel context (context A or context B) defined by type of bedding and gnawing block size and shape. Bedding materials used were beech (context A, P.P.H. "WO-JAR", Poland or PPHU Natur-Drew A. Czaja, Poland or Terrario Peak Wilderness, DMR Group, Poland) and cellulose (context B, Scott Pharma Solutions, cat no. L0107). In the home cages, aspen bedding was used (ABEDD, Latvia or Tapvei GLP, Estonia). Mice were allowed to freely explore the test cage for 30 minutes, and the time spent in each compartment

was recorded. Animals that spent more than 70% of the pretest time in one of the contexts were excluded (**Table S2**).

After the pretest, animals were returned to their home cages for approximately 24 h. Then, mice were assigned to undergo social conditioning (housing with cage mates) for 24 h in one of the contexts used in the pretest followed by 24 h of isolate conditioning (single housing) in the other context. Conditioning was performed in cages identical to the home cage, with ad libitum access to food and water. To prevent bias (**Fig. S1A**), the social context was randomly assigned such that approximately half of the animals received social conditioning in context A and half in context B. In cases where the final number of animals conditioned in each context was not equal (due to an unequal number of animals passing the 70% criterion or unequal number of animals in the litter), we pseudorandomly trimmed the larger group using a Python script (https://zenodo.org/record/8100281). The exception from completely random selection was introduced to preserve a mean 50% initial context preference during the pretest (for details, see Supplementary Materials). The conditioning phase lasted 6 days (3 days in each context, alternating every 24 h), and then the posttest was performed.

Two measures of the rewarding effects of social interactions were used: 1) pretest vs. posttest comparison of the time spent in the social context, 2) score: time spent in the social context minus time spent in the isolation context during the posttest.

Opioid antagonist administration

To block mu-opioid receptors, animals were given i.p. injection of cyprodime hydrochloride 1 hour before the posttest (TOCRIS, cat. no. 2601, dissolved in saline, 1 mg/kg, 5 µl/g). A previous experiment demonstrated that saline injection before the posttest did not influence sCPP expression (**Fig. S1B**).

Social interaction in the partitioned cage

This test was carried out to assess social contact seeking with a sibling partner after 24 h of isolation. The procedure was performed in a rectangular cage (48 × 12 cm, 25 cm high) divided by a transparent, perforated plastic wall into two compartments: a smaller partner compartment and a larger focal animal compartment (**Fig. 2A**). One day before the test, the animals were weighed, and the heavier animal from each pair was designated as the focal animal. Next, the animals were habituated to their respective cage compartments for 10 minutes. During habituation, only one mouse was present in the test cage. After habituation, mice were placed in separate home cages for approximately 24 h, after which time the focal animals were placed in the test cage for the second adaptation session (5 minutes). After adaptation, the partner was introduced for 10 minutes. Two measures of social contact seeking were used: time spent in close proximity to the partner's compartment and distance to the partner's compartment.

Cocaine-induced conditioned place preference

For the CPP paradigm, three-compartment cages were used; the two peripheral compartments (that contained distinctive visual and tactile cues) were linked to the central compartment by guillotine doors (Med Associates, St. Albans, VT, USAMED-CPP-MSAT). The test consisted of three phases: pretest, conditioning, and posttest. For the pretest and posttest phases, animals were introduced to the central compartment of the apparatus, and the doors between the compartments were lifted such that the animals could freely explore the apparatus for 20 minutes. Animals that spent more

than 70% of the pretest time in one of the contexts were excluded. The less preferred of the two peripheral compartments was designated the cocaine compartment. The next day, the 3-day conditioning phase started. Each day, two 40-minute conditioning sessions were performed, separated by approximately 3 hours, during which animals were kept in their home cages. Before the morning conditioning session, animals received an i.p. saline injection, while before the afternoon session, they were injected with cocaine hydrochloride dissolved in saline (10 mg/kg, 5 µl/g). Immediately after the injection, animals were placed in the respective cage compartment. The posttest was performed on the day after the last conditioning session. To match the manipulation of mice during the sCPP experiments with that during the cocaine-induced CPP, animals were injected with saline (5 µl/g) one hour before the posttest.

Data analysis

The distance traveled and time spent in separate cage compartments in the sCPP and social interaction tests were analyzed automatically using EthoVision XT 15 software (Noldus, The Netherlands). In the social interaction test, the zone close to the partner's compartment was outlined digitally. In the cocaine-induced CPP test, the position of the mouse was registered automatically by the Med Associates system. Comparisons of sample means were performed using analysis of variance (ANOVA) followed by Sidak's post hoc correction or Student's t test for cases with only two samples. The statistical significance threshold was set at $p < 0.05$. Before the analysis, the Grubbs test for outliers was performed. The analysis was performed in GraphPad Prism 9.4.1. For full descriptive statistics and statistical test results, see **Supplementary Materials**.

Acknowledgments
Funding: Polish National Science Centre grant 2016/21/B/NZ4/00198.
Author contributions: Conceptualization: ZH, BZ, JRP; Methodology: ZH and JRP; Investigation: ZH, MK, KM, MC, ŁS, and MKJ; Visualization: ZH and JRP; Supervision: BZ, JRP; Writing—original draft: ZH and BZ; Writing—review & editing: ZH, BZ, RR, and JRP.
Competing interests: The authors declare that they have no competing interests.
Data and materials availability: All data are available at https://zenodo.org/record/
We would like to thank Dr. Jakub Dzik from the Nencki Institute for Experimental Biology, Polish Academy of Sciences for writing the Python script used for social conditioned place preference data trimming (https://zenodo.org/record/8100281).

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Figures

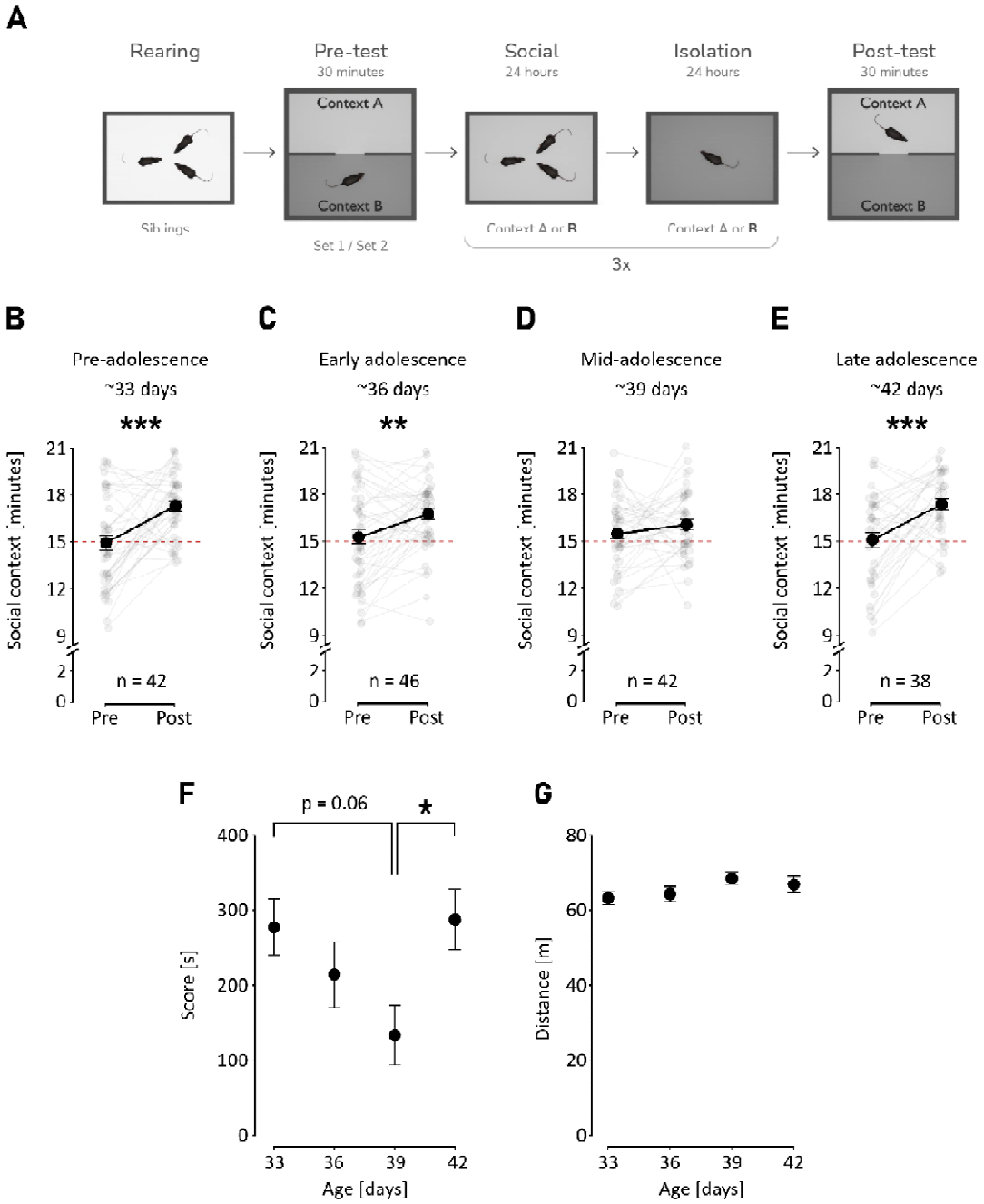

Fig. 1. Social conditioned place preference during adolescence. (**A**) Schematic representation of the experimental schedule. Male C57BL/6 mice were housed and tested in sibling groups. (**B-E**) Mice at approximately postnatal days 33 (B, n = 42), 36 (C, n = 46) and 42 (E, n = 42) but not postnatal day 39 (D, n = 38) at posttest showed a robust conditioning effect, measured as an increase in the time spent in social context from pretest to posttest (paired t test, P33; t_{41}=4.45, p<0.001, P36; t_{45}=3.17,

p=0.003, P39; t_{41}=1.19, p=0.24, P42, t_{37}=4.05, p<0.001). (**F**) The social preference score was lower at P39 than at P33 and P42 (ANOVA, $F_{3,164}$ = 2.98, p = 0.063, Sidak's post hoc, P33 vs. P39, p = 0.063, P39 vs. P42, p = 0.049). (**G**) The distance traveled during posttest did not differ among the groups (ANOVA, $F_{3,164}$ = 1.645, p = 0.181).

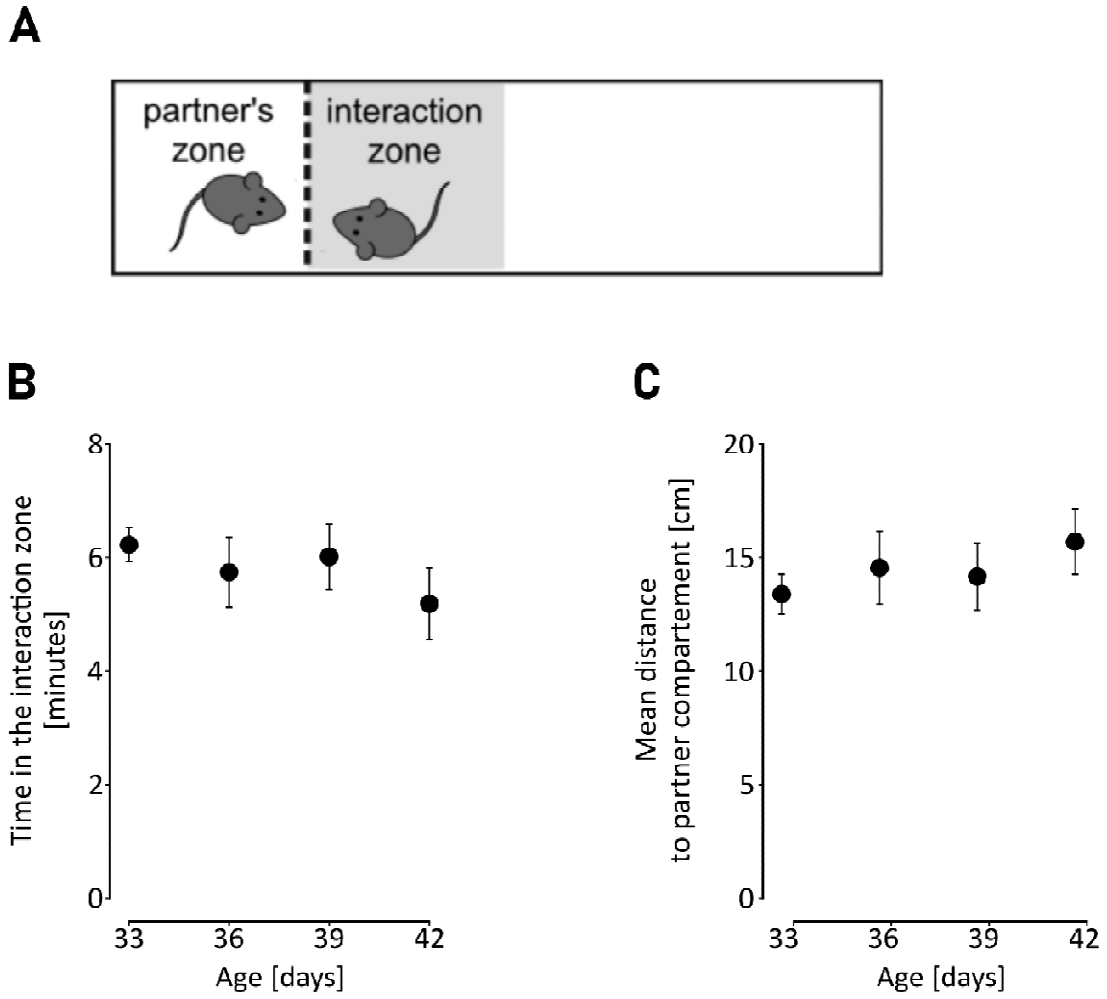

Fig. 2. Social contact seeking during adolescence. (**A**) Summary of the procedure. Mice were tested for seeking social contact with a sibling after 24 h of isolation. (**B**) No changes in time spent in proximity to the partner were observed during adolescence (ANOVA, $F_{3,34}$ = 0.72, p = 0.55). (**C**) No changes in proximity to the partner's compartment were observed during adolescence. One-way ANOVA revealed no significant effect of age on either parameter. n = 9/10 per group (ANOVA, $F_{3,34}$ = 0.52, p = 0.672).

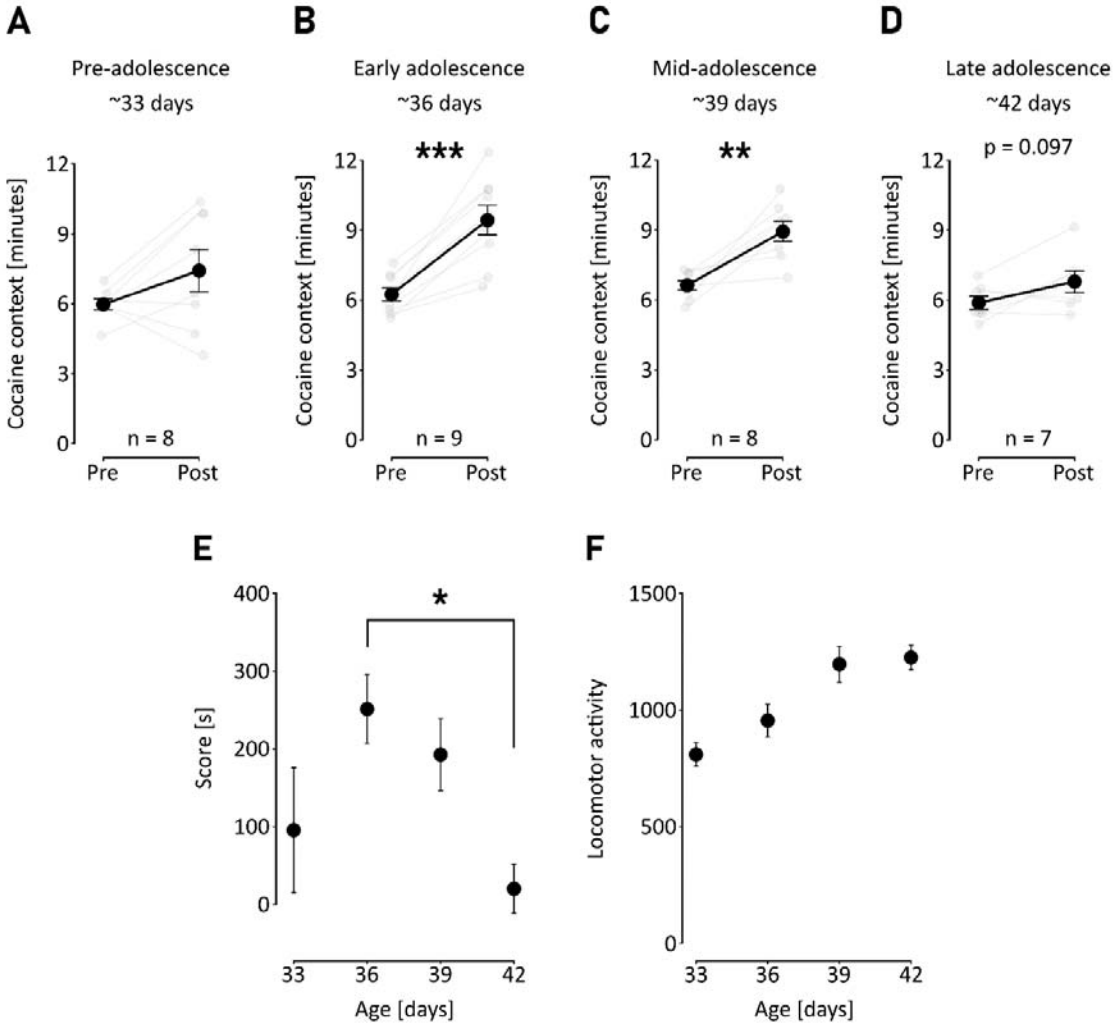

Fig. 3. Cocaine-induced conditioned place preference during adolescence. (**A-D**) Mice aged 36 (B, n = 9) and 39 (C, n = 8) but not 33 (A, n = 8) or 42 (D, n = 7) days at posttest showed a robust conditioning effect, measured as an increase in the time spent in the cocaine context from pretest to posttest (paired t test, P 33; t_7=1.76, p=0.12, P 36; t_8=6.44, p<0.001, P 39; t_7=0.4.34, p=0.003, P 42, t_6=1.98, p=0.097). (**E**) The cocaine preference index was higher at P36 than at P42. However, the result did not reach statistical significance (one-way ANOVA, $F_{3,28}$ = 1.98, p = 0.056, Sidak's post hoc test, P 36 vs. P 42, p = 0.056). (**F**) The cocaine preference score was significantly higher at P36 than at P42 (one-way ANOVA, $F_{3,28}$ = 3.48, p = 0.029, Sidak's post hoc test, P36 vs. P42, p = 0.029). (**G**) The distance traveled increased with age (ANOVA, $F_{3,28}$ = 9.17, p < 0.001).

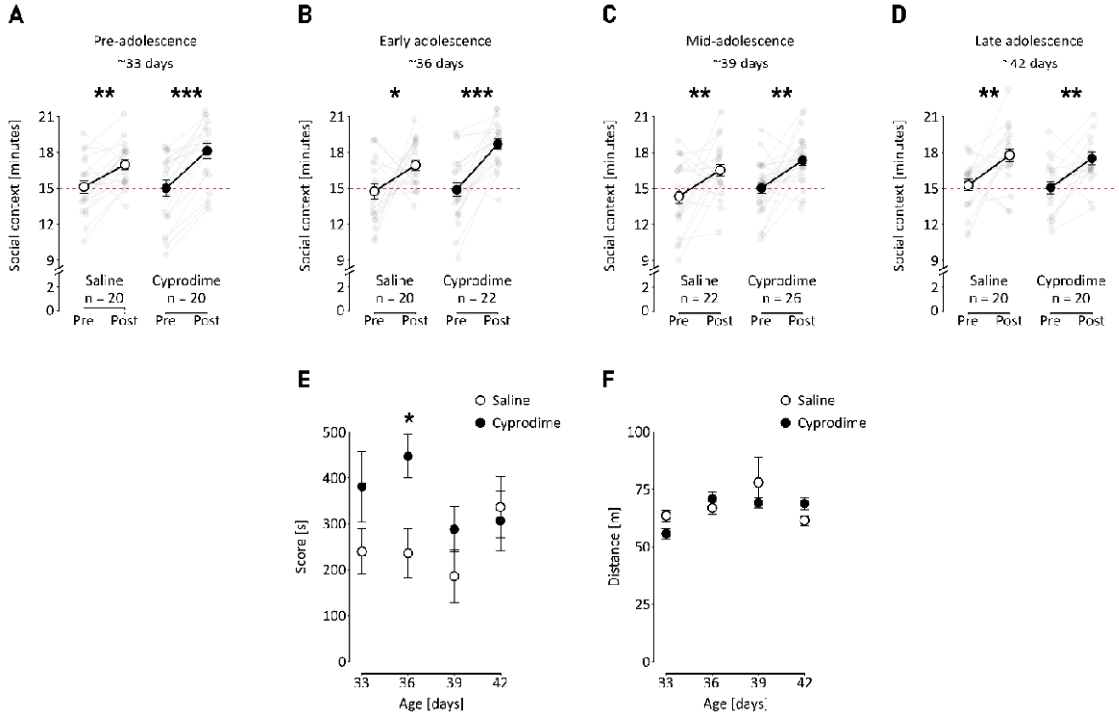

Fig. 4. Influence of the mu-opioid receptor antagonist, cyprodime, on the expression of social conditioned place preference during adolescence. (**A-D**) Cyprodime significantly increased sCPP expression in animals aged 36 days (B, saline, n = 20, cyprodime, n = 22) and had no effect in mice aged 33 (A, saline, n = 20, cyprodime, n = 20), 39 (C, saline, n = 22, cyprodime, n = 26) or 42 days (C, saline, n = 20, cyprodime, n = 20) as determined by two-way repeated-measures ANOVA (drug effect: P33, $F_{1,38} = 0.552$, $p = 0.462$; P36, $F_{1,40} = 4.1$, $p = 0.05$, P39, $F_{1,46} = 2.44$, $p = 0.125$, P42, $F_{1,38} = 0.222$, $p = 0.64$). (**E**) Cyprodime increased the social preference score. This effect was mainly due to the increase observed in P36 mice but not in younger or older animals, as revealed by two-way ANOVA (drug effect, $F_{1,162} = 6.571$, $p = 0.011$, Sidak's post hoc, P33, $p = 0.34$, P36, $p = 0.04$, P39, $p = 0.572$, P42, $p > 0.994$). (**F**) Cyprodime had no effect on the distance traveled during posttest (interaction between drug and age: $F_{3,162} = 1.539$, $p = 0.207$, age: $F_{3,162} = 3.233$, $p = 0.024$, drug: $F_{1,162} = 0.149$, $p = 0.699$).