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Using maternal rescue of pups in a cup to investigate mother-infant interactions in mice/rodents

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Highlights

- The behavioural assay of "maternal rescue of pups in a cup" investigates mother-pup interactions
- Pups' calmness as well as maternal retrieving tactics significantly contribute to the efficiency of maternal retrieving
- Experimental perturbation of pups' sensory-motor system induces readjustment of maternal tactics

Keywords

- Transport response; maternal rescue of pups in a cup; mother-pup interactions

Abstract

Efficient parental care is indispensable for survival of the mammalian offspring, and therefore both parents and offspring cooperate to achieve the best performance. For example, when parents transport altricial offspring, the offspring immediately respond by reducing its cry and movement in both human infants and rodent pups. This coordinated set of central, motor and cardiac responses is designated as the Transport Response (TR) and is shown to facilitate maternal carrying in rodents. The present study aims to investigate the core behavioural characteristics of mother-infant interaction, and to investigate the mechanisms underlying the mother-pup cooperation using pharmacological and genetic manipulations (i.e. *Oprm1-/*). Along with the clear developmental changes of the pups' immobility and posture during maternal carrying as previously reported, there were also adaptations in maternal strategies, particularly in positioning of foothold and oral grasp over the pup's body, with the pups' age and pup's behaviour. Tree-based models elucidated that both of these maternal variables as well as percentage of pups' struggle predict the time required for pup retrieval from a cup. When the sensory-motor control in pups was disturbed by pharmacological or genetic manipulations, these core behaviours were inefficiently performed and impede maternal retrieval. Mother-infant mutual fit is a complex construct where several intermingled mechanisms are involved. Thus mothers and infants, when interacting, should be considered together as one whole system in which any change in one side or the other, affects the output of the whole dyad. The outcome of the interaction relays on a specific dynamic pattern of infant and maternal behaviours, which mutually change and adapt to fit each other's needs. Key features to reach a successful outcome of the interaction were the maternal retrieving strategy and infants' Transport Response behaviour.

1. Introduction

Mammalian parents and offspring cooperate to achieve the survival and development of the offspring (Bowlby, 1969; Insel, 2001; Hofer, 1996). This primitive and yet critical social relationship is conserved among mammals, and shapes the social behaviours in the later life of offspring. In modern neuroscience, however, the parent-infant interaction has not been attracting enough attention as it should deserve. This may be due to the technical difficulties of observing the swift and complex parent-offspring interactions, especially as the offspring's behaviour of small mammals are difficult to observe quantitatively, and to dissect neurologically.

We have recently established a cooperative calming response in infants during maternal carrying. Originally, several researchers described that maternal oral transport induces a characteristic compact posture in carried infants of many quadruped mammals (Eibl-Eibesfeldt, 1951; Sauer, 1967; Schaller, 1972), and this postural regulation has been studied experimentally in laboratory rats and named as "Transport Response" (Brewster and Leon, 1980; Wilson et al., 1984). We have confirmed their findings in laboratory mouse pups, and further identified the same set of calming responses (immobility, reduction of heart rate and distress vocalizations) in human infants and mouse pups (Esposito et al., 2013; Gammie, 2013). These calming responses observed in human infants and the young of non-human mammals, including postural regulation described above, are collectively called the "Transport Response" (TR) in this article.

Mouse TR emerges transiently before weaning, reaching a peak in the second postnatal week and gradual attenuation from postnatal day (PND) 15, and coincides with the maturation of pups' visual and ambulatory ability (Yoshida et al., 2013). The TR can be easily induced by picking the pup up and lightly pinching the skin on the back of the pup's neck with the experimenters' fingers to mimic maternal oral grasping (Brewster and Leon, 1980; Esposito et al., 2013; Wilson et al., 1984). Somatosensory and proprioceptive inputs are both required for induction, such that when the pups' proprioception was inhibited, the pups struggled even during maternal carrying, and retarded maternal rescue significantly.

Therefore, the function of TR seems to facilitate maternal carrying (Esposito et al., 2013; Esposito et al. 2015). Taken together, the TR can be regarded as a primitive attachment behaviour to maintain maternal proximity.

During the course of the previous studies, we invented a new behavioural task, called "maternal rescue of pups in a cup" for three reasons; first, during maternal oral carrying of pups in the normal cage, it is hard to observe pups' behaviour clearly; second, in a small and flat home cage, pup retrieval is too easy for the mother and doesn't need much of the pup's contribution; and third, pups become able to crawl back to the nest using olfactory cues after PND 7 and mothers tend to refrain from oral retrieval afterwards. Therefore, we placed a transparent cup in the home cage and put the pups into it. In this setting, pups are not able to get out from the cup and go back to the nest by themselves, until they become able to see after eyelid opening around PND 14. This new setting enabled us to observe the tactics and contribution of both mothers and pups to facilitate the rescue task in more detail, and for much longer (until the third postnatal week). The present study first aims to describe the core behavioural characteristics of mothers and pups, and their typical postnatal development in congenic C57BL/6J mice, and their effects on the efficacy of maternal oral rescue of pups as manifested in terms of retrieving time. This study also aim to give methodological details and tips to help other researchers to test mother-infant interaction in a similar setting in mice, rats or small mammals.

As models for atypical mother-pup cooperation, we used various pharmacological and genetic manipulations, including a) anaesthetized pups, b) *Oprm1-/-* pups lacking the gene encoding mu-opioid receptor (Matthes et al., 1996) and c) *Oprm1-/-* postpartum female mice, d) pups carrying homozygous *reeler* mutation, which leads to severe hypoplasia of the cerebellar cortex (D'Arcangelo et al., 1995). The mu-opioid receptors have been implicated in social behaviours and isolation distress in a wide range of animals and humans (Pert & Snyder, 1973; Hsu et al., 2013). Mice lacking the *OPRM1* gene encoding for mu-opioid receptor are generally healthy and fertile, but are shown to exhibit numerous autistic-like phenotypes, such as fewer ultrasonic vocalizations upon maternal separation by pups

(Moles et al., 2004), lower interest in peers by juveniles (Cinque et al., 2012) as well as adults (Becker et al., 2014; Wohr et al., 2011), decreased social aversion after repeated social defeat stress (Komatsu et al., 2011), and increased stereotypy (Becker et al., 2014), compared with their wild-type controls. Also, in a study performed with human subjects by Way & colleagues (2009), distinct allelic forms of the *OPRM1* gene differentially moderated participants' sensitivity to social pain. We therefore selected *Oprm1-/-* mothers and pups to be examined their interaction using our "maternal rescue of pups in a cup" assay. Additionally, the cerebellum is heavily recruited in motor control and coordination. Pups carrying homozygous *reeler* mutation that causes a lack of reelin, an extracellular protein involved in corticogenesis, are affected by severe cerebellum hypoplasia (Mariani et al., 1977; D'Arcangelo et al., 1995). *Reeler* mutant *rl/rl* pups display abnormal posture during TR without showing differences in body-weight and general appearance from the wild-type pups (Esposito et al., 2013). We therefore examined *reeler* mutant pups to investigate the role of motor coordination in mother-offspring cooperation during our "maternal rescue of pups in a cup" assay.

2. Methods and Measurements

2.1 Animals

All animal experiments were approved by the RIKEN Wako Animal Experiments Committee as complying with the ARRIVE guideline, and were conducted in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). C57BL/6 mice were purchased from Japan SLC and CLEA Japan. B6.129S2-*Oprm1*$^{tm1Kff/J}$ were obtained from the Jackson Laboratory and genotyped as previously described (Esposito et al., 2013). B6C3Fe *a*/a-*Reln*$^{rl/J}$ (*reeler*) mutant line was originally obtained from the Jackson Laboratory and maintained within the mutant colony of Dr. Masaharu Ogawa's laboratory, and then provided to our laboratory. The *reeler* mutant mice were maintained and analysed in the mixed background, without

157 backcrossing into C57BL/6 background, because the homozygous mutants were too
158 physically weak to be tested in the C57BL/6 background as described previously (Caviness
159 et al., 1972). This difference of genetic background caused the increase of pup growth
160 compared with C57BL/6 pups, and thus tested slightly earlier as shown in Fig. 6. This
161 difference of the genetic background should be taken into account to compare the *reeler*
162 results with other results in this study (Wolfer et al., 2002; Hiroi, 2018).

163 Mice were maintained under a 12-h light/dark cycle (lights-on 08:00) with food and
164 water ad libitum. C57BL/6 pups were culled to six by PND 4, but without cross-fostering. For
165 genetic mutant pups, each mutant pup received the injection of animal tattoo ink (Natsume,
166 Japan) into the forepaw or the footpad to distinguish each other and a small piece of the tail
167 were collected for genotyping PCR at PND 3 or 4. The number of pups of mutant mouse
168 lines was culled to six at PND 5, but no cross-fostering was adopted. Genotyping was
169 performed twice for each animal to verify the result, once before culling and another after the
170 experiments. Only non-primiparous mothers were tested in order to avoid the variance due
171 to lack of experience.

172

173 **2.2 Behavioural task of "Maternal rescue of pups in a cup"**

174 The behavioural task "maternal rescue of pups in a cup" was performed in a home cage of
175 standard shoebox size (approx. size of 265mm x 205mm, 140mm high) with individual
176 ventilation, the common practice for mutant mouse husbandry in a specific-pathogen-free
177 condition. In preparatory analyses, we tried several commercially-available cups made of
178 glass or polypropylene. It turned out that C57BL/6J mothers were not able to stand and carry
179 the pups securely at the top of the cup if the rim was without a hem or ridge. Therefore we
180 chose a screw-top polypropylene cup (590 ml, top diameter = 125 mm, bottom diameter = 90
181 mm, inside height = 85 mm (EveryPack Round No. 3, Sanko Plastics Co., Mie, Japan). It
182 should be noted that although the cup without a lid was used as it is in this study, the cup
183 was later transected perpendicularly at 25 mm from the center and fixed with its open end to

the cage wall in order to obtain the best visibility of subject mice from the camera (see Yoshida et al., 2013). In this case, an additional triangular cut at the bottom of open end was added to fit the cup to the cage wall, the bottom part of which was rounded.

The above described cup was fixed in the home cage [Fig.1A-B] of C57BL/6 mouse mothers and their litters at PND 4 (5 hours before testing), and the mothers were put in the cup for three times to habituate them to the cup. Then, at PND 4, 8, 12 and 16, test sessions were started by taking three pups out of the nest and placing them into the cup. After the first session, another three pups were introduced into the cup for the second session. These rescue sessions were video recorded using a Handycam HDR-SR12 (Sony), and the various parameters of the pups' responses to the maternal retrieving behaviour were measured by a frame-by-frame video-analysis (25 frames per second) using the Free Video To JPG Converter (DVDVideoSoft, IL).

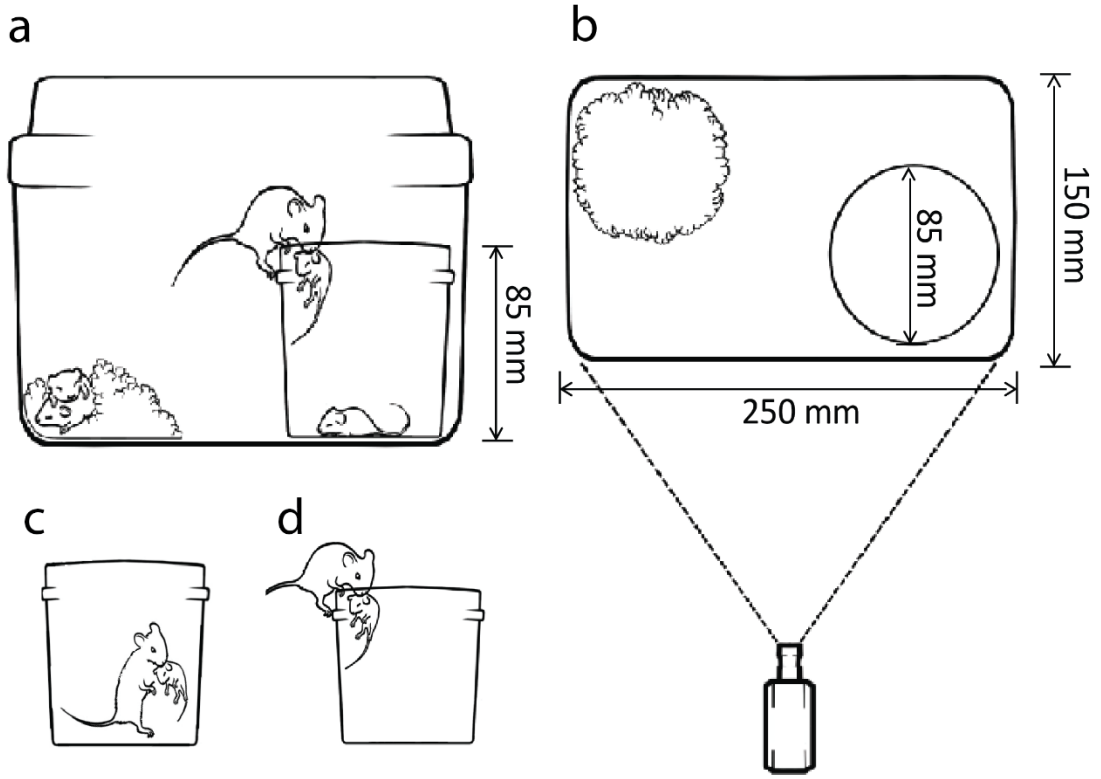

Figure 1. Experimental setting. a) Representation of the lateral view of the cage during an experimental session. In the left corner the nest is visible with the already rescued pups. In the right corner the cup where the pups were left when separated by the mother is shown. On the top of the cup a mother retrieving one pup is represented. b) Representation of the upper view of the cage. In the upper left corner the nest is visible, while in the lower right corner the cup is shown. Experimental sessions were recorded with a camera (showed here) placed in order to have a lateral point of view of the entire side of the cage – view shown in panel (a). c) Representation of a mother while retrieving a pup at the bottom of the cup. d) Representation of a mother while retrieving a pup from the ridge of the cup.

2.3 Behavioural variables

Maternal behaviour

Retrieving position. A mother places her hind limbs at the cup bottom when she grasps the pup and then climbs up the cup wall (inside), or keeps her hind limbs on the top ridge of the cup while grasping the pup (from the ridge).

Grasp. Pups' body area where mothers bite to transport the pup: (i) neck, (ii) mid backbone, (iii) end backbone, (iv) hip or belly, (v) head, limbs or tail.

Pups' Behaviours

Passive Motion. The pup is immobile with all limbs extended. Calculated as percentage over the total time.

Partial TR. The pup is immobile with at least one flexed limb. Calculated as percentage over the total time.

TR. The pup is immobile with all limbs flexed. Calculated as percentage over the total time.

Struggling. The pup show rapid and anti-gravitational movements – the direction of movements is opposed to gravity direction – throughout the body. Calculated as percentage

over the total time. The passive movement of the pup caused by the maternal movement was not regarded as pup movement.

Session-related variables

Retrieving time. Time from when the mother orally grasped the pup until when the entire body of both the mother and the pup were out of the cup and the mother's four limbs touched the ground. Only successful maternal attempts to retrieve the pups were assessed for retrieval time.

2.4 Statistical Analysis

Maternal Behaviours. To examine the effect of each explanatory variable (postnatal days as a continuous variable, and the pharmacological or genetic manipulation as a categorical variable), binary logistic regression analysis was conducted with maternal rescue position (2 categories; inside vs ridge of the cup) as a response variable, and multinomial logistic regression analysis were conducted with maternal grasp (5 categories; neck, middle of backbone, end of backbone, hips or belly, and head, limbs or tail) as a response variable. We selected the best fitted model using Akaike's Information Criterion (AIC), then conducted Wald test to find significant variables and interaction. If there was any significant interaction found, we divided the data by PND and conducted the same procedure to find significant simple main effects of pharmacological or genetic manipulation at each PND.

Pups' Behaviours. Linear model regression analysis was conducted to examine the effect of each variable on pups' behaviours. We calculated the proportion of time each pup spent performing each of the 4 different behaviours (passive motion, partial transport, TR, and struggle) to the total observation time and used them as response variables. Then the same procedure with AIC and Wald test explained above was conducted.

Retrieving Time. Linear model regression analysis was conducted to examine the effect of each variable on the retrieving time. The same procedure with AIC and Wald test explained above was conducted.

General Linear Model. To elucidate the contribution of pups' (PND, body weight, TR, struggle) and maternal (maternal position and grasp) variables to retrieving efficacy, a general linear model with the retrieving times as dependent variable was performed. Since not all the pups performed a full TR and considering that only partial TR is present in the first PNDs, the full and the partial TR values were summed in a one combined TR variable in this model. First, the best model was selected applying the stepwise selection – stepAIC() function in R – in both directions, forward and backward. The resulting best model featured as independent variables three pups' characteristics (PND, combined TR, struggle) and one maternal characteristic (maternal position). Then, to better evaluate the effects of the combined variables for each behaviour (combined TR, struggle), pups were subdivided in high vs low frequency subgroups using the median split procedure. Students' t test were then applied as post-hoc tests in order to clarify the direction of the combined effect of pups' behaviour and maternal position on retrieving times.

Tree-Based Model. To determine which characteristics and behaviours were operative in favoring maternal caregiving (retrieving time) in mice, we employed recursive partitioning, specifically regression tree models (Costello et al., 2003), in an exploratory way. Recursive partitioning explores the structure of a data set while developing easy-to-visualize decision rules for predicting a continuous (regression tree) outcome. Regression tree or tree-based models consist of two main steps: growing (exploring relations among variables) and pruning (minimizing overfitting the data). That is, the model first explores all possible relations among variables and then evaluates which values of the independent variables stratify the dependent variable in sample pairs that differ statistically. Tree-based models provide information about the (1) hierarchy of importance of independent variables in explaining the distribution of data points of the dependent variable and (2) which value ("node") of the

independent variable subdivides (“splits”) the dependent variable in sample pairs that differ statistically. The whole database, regardless of pharmacological or genetic manipulations, was used to fit the model. Since not all the pups performed a full TR and considering that only partial TR is present in the first PNDs, the full and the partial TR values were summed in a one combined TR variable in this model. Statistical analyses were conducted using R version 3.4.4 and 3.3.2 (R Core Team, 2018). R package nnet version 7.3.1 was used for the multinomial logistic regression analysis (Venables & Ripley, 2002), and rpart version 4.1.13 was used for the Tree-Based Model (Therneau & Atkinson, 2018).

3. Typical Development

At PND 4, 8, 12 and 16, the behaviour of both the wild-type C57BL/6J mothers and pups during the "maternal rescue of pups from a cup" sessions [Fig.1A] were coded and analyzed [Fig.2]. The same animals were tested in the different PNDs. From PND 4 to 16, all pups were typically retrieved by the mother within 15 minutes. After PND 17 pups were able to escape from the cup without maternal help (Yoshida et al., 2013). Therefore, no pups remained in the cup throughout the experiments. It should also be noted that when rescuing the pup from the cup, all of the mothers kept holding each pup when they got out of the cup and never dropped the pup out of the cup while they still remained on the ridge of the cup.

Results

Maternal behaviours. At each successful pup rescue, the mother orally grabbed the pup either from the top ridge (Fig. 1d) or at the bottom floor of the cup (Fig. 1c). The logistic model showed a significant effect of PND on maternal positioning during successful retrieval, $p < .01$ [Fig.2A, Table 1 of supplementary materials]. Specifically, maternal retrieving from the cup ridge increased steadily in subsequent PNDs while retrieving from inside decreased

steadily. Also, a significant effect of PND was found on the distribution of pups' body parts grasped by the mother [Fig.2B, Table 2 of the supplementary materials]. Grasping from the neck became more frequent in subsequent PNDs compared to the use of mid-back, end-back, or head/tail/limbs grasps which decreased steadily over time (Mid back: $p < .05$; End back: $p < .05$; Head/tail/limbs: $p < .001$). No significant effect of PND was found on neck vs belly/hips grasps.

Pups' behaviours. As partly presented previously (Esposito et al., 2013), PND has a significant effect on the distribution of passive motion, partial transport and TR behaviours [Fig.2C, table 3 of supplementary materials]. Specifically, PND had a significant effect with passive motion decreasing steadily over time and partial transport and TR increasing steadily over time (Passive motion: $p > .001$; Partial TR: $p > .001$; TR: $p > .001$).

Retrieving time. A significant main effect of age was found on retrieval time, $p < .01$ [Fig.2D, Table 4 of the supplementary materials].

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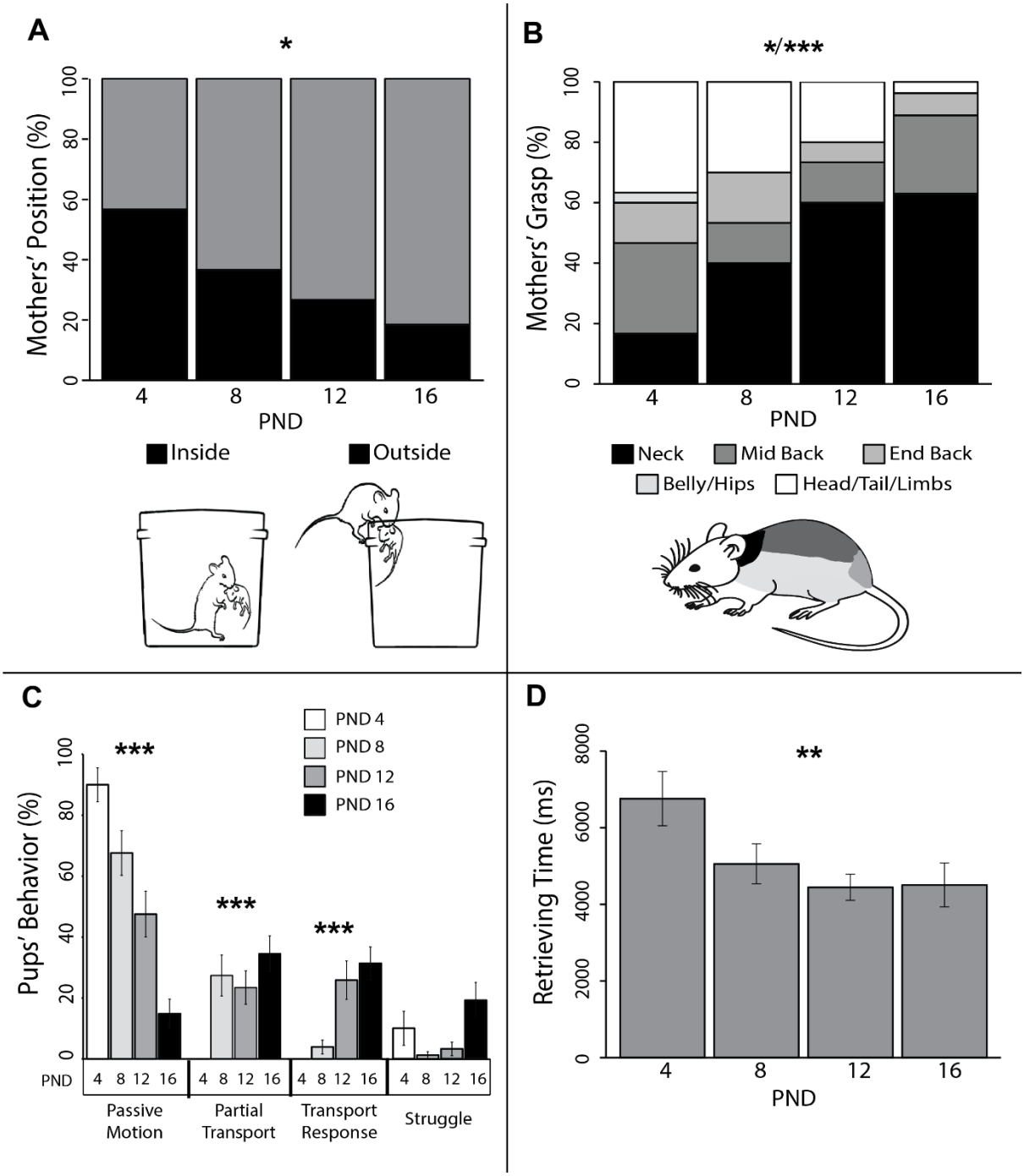


324

325 **Figure 2.** Mothers' and pups' typical behaviours during the retrieving test in different Post-
326 Natal Days (PNDs). The same animals were tested in the different PNDs. N=30 for PND 4,
327 8, 12, and N=27 for PND 16. A) Percentage of retrieving from inside vs outside the cup. *
328 indicates the main effect of PND on maternal position. B) Percentage of used maternal
329 grasp: neck, mid back, end of back, hips or belly, and head or tail or limbs. Here the
330 multinomial logistic regression produced different significances of the PND effect for each

category (body part). * indicates the main effect of PND on mid back vs neck grasp and on end of back vs neck grasp. *** indicates the main effect of PND on head, limbs, or tail vs neck grasp. See the supplementary materials for more details. C) Time (% of seconds) spent by pups displaying different behaviours: passive motion, partial TR, TR, and struggle in different PNDs. *** indicates the main effect of PND on pups' passive motion, partial TR and TR. D) The mean ± S.E.M. retrieving time at each test day. ** indicates the main effect of PND on retrieving times. * $p < .05$, ** $p < .01$, *** $p < .001$

General linear model. To evaluate the contribution of each maternal and pup's variable to the efficacy of rescue as indexed by the retrieving time, the General Linear Model was applied. Significant effects of PND, combined TR, struggle behaviours, and maternal position (from inside vs from the ridge of the cup) were found on retrieving times (PND: $t = -3.03$, $p < .01$; Combined TR: $t(57)= 2.84$, $p < .01$; Struggle: $t(57)= 4.55$, $p < .001$; Maternal position: $t= -3.18$, $p < .01$).

To better understand the role played by pups' behaviours in determining the outcome of the interaction, the group was split in high vs low combined TR and high vs low struggle behaviours by applying the median split procedure and the retrieving times were compared between the two groups. Analysis showed that the retrieving times are significantly lower when the combined TR behaviour is higher ($t(115)= -2.05$, $p < .05$) [Table1].

	Mean(SEM)		**df**	***t* value**	***p* value**
	Low	**High**			
Combined TR	5783.32(469.09)	4627.97(310.58)	115	-2.05	<.05
Struggle	4997.06(298.11)	6466.59(856.97)	115	1.83	ns

Table 1. In the table the mean and S.E.M retrieving time when pups' show high vs low combined TR response or struggle behaviours are reported.

4. Pharmacological Manipulations

Esposito, Yoshida and colleagues (Esposito et al., 2013) have shown that two components of pups' TR, one being immobilization and another being compact posture, contribute to the efficacy of maternal rescue of the pup from a cup. To further evaluate the pups' behavioural contribution to the mother-pup interaction during maternal carrying, a general anaesthetic pentobarbital was applied at two different concentrations to PNDs 8 and 12 pups. Intraperitoneal injection of 40mg/kg sodium pentobarbital (Kyoritsu Seiyaku, Japan) made the pups completely immobile and non-responsive to painful stimuli (Esposito et al., 2013). On the other hand, the dose at 30mg/kg pentobarbital, pups did not show proper TR and rather struggle by manual carrying by experimenter in the preparatory experiment. The control group received a saline injection, which was a mild stress by itself. All injections were performed 10 min prior to the test session. The same animals were tested in the different PNDs and therefore each animal received the injection twice, once at PND 8 and once at PND 12.

Results

Maternal behaviours. A significant effect of anaesthesia was found in the complete anaesthesia (A40) group on the distribution of maternal position, $p < .01$ [Fig.3A, Table 5 of the supplementary materials]. Mothers in the A40 group showed an overall increase of retrieval from inside the cup compared to the mothers in the control group. Concerning maternal grasp during retrieval, mothers in the partial anaesthesia group increased the use of head/tail/limbs grasping across PNDs compared to control group, $p < .05$ [Fig.3B, Table 6 of the supplementary materials]. These data suggest that the healthy pups contribute to maternal grasp at the preferred position (neck or mid back). This behaviour has been reported also in several other mammalian species (see Discussions section).

381 *Pups' Behaviours*. Analysis showed significant effects of PND and anaesthesia group on
382 pup's behaviours [Fig.3C, Table 7 of the supplementary materials]. At PND 8 pups in the
383 partial anaesthesia group showed more struggle and less passive motion compared to
384 control pups, while the pups in the complete anaesthesia group almost always showed
385 passive motion, as expected. Partial TR and TR only occurred in the control group and,
386 within the control group, TR increased in PND 12 compared to PND 8.
387
388 *Retrieving Time*. A significant main effect of the anaesthesia group was found on retrieval
389 time, $p<.001$) [Fig.3D, Table 8 of the supplementary materials]. Specifically, in the group
390 where pups had received the complete anaesthesia (40mg/kg), retrieving times were higher
391 compared to the control group at both PNDs. Therefore, in concordance with previous
392 findings (Brewster and Leon, 1980; Esposito et al., 2013), it was concluded that maternal
393 carrying efficacy was hindered when the pups were completely anaesthetized.
394

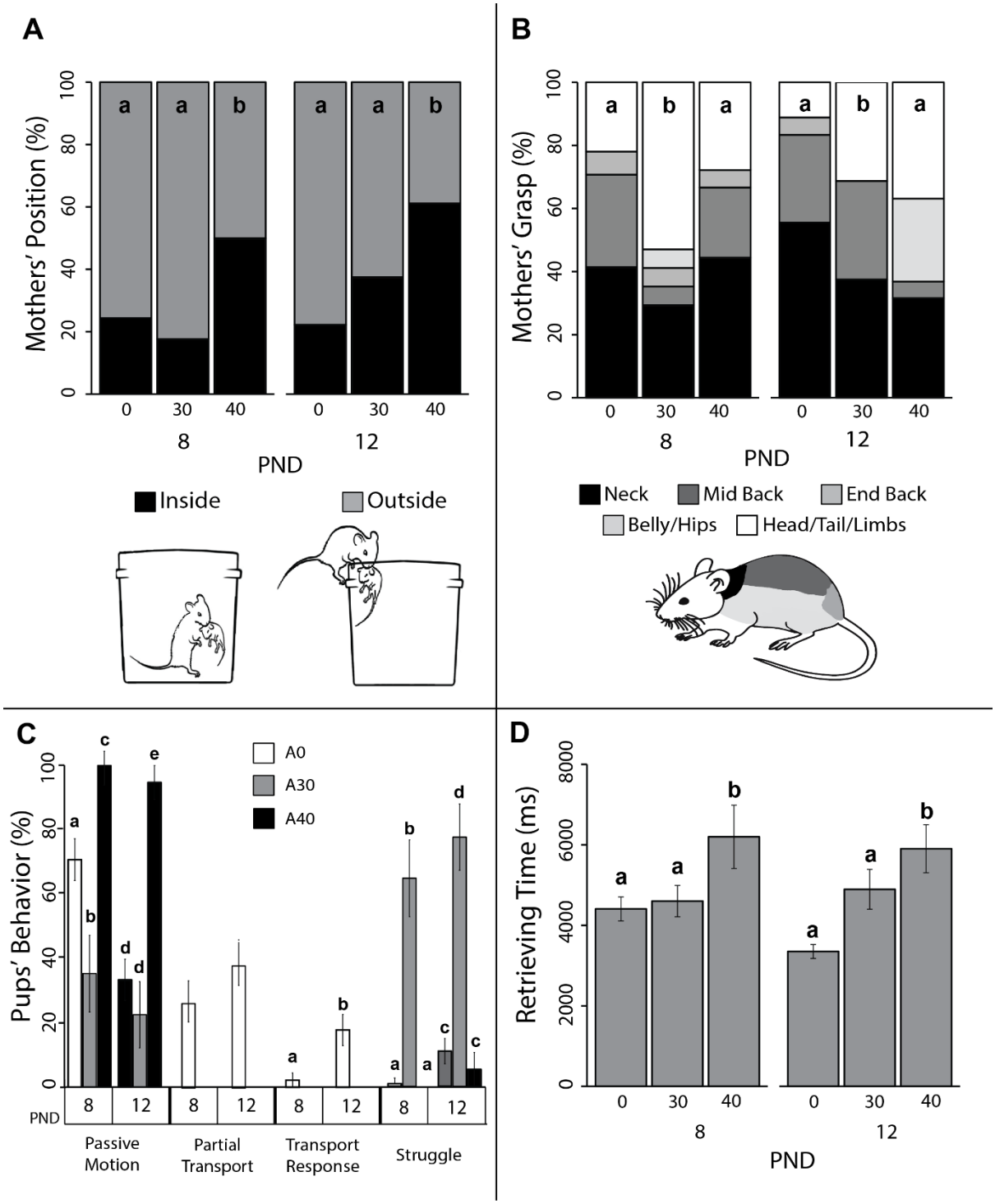


Figure 3. Mothers' and pups' behaviours during the retrieving test while pups were anaesthetized with sodium pentobarbital. Three different anaesthesia levels – 0mg/kg (saline) (N=41 for PND 8, N=36 for PND 12), 30mg/kg (N=17 for PND 8, N=16 for PND 12), 40mg/kg – are shown (N=18 for PND 8, N=19 for PND 12). The same animals were tested in the different PNDs and therefore each animal received the injection twice. A) Percentage of

retrieving from inside vs outside the cup. B) Percentage of used maternal grasp: neck, mid back, end of back, hips or belly, and head or tail or limbs. C) Time (% of seconds) spent by pups displaying different behaviours: passive motion, partial TR, TR, and struggle. PNDs are represented on the x axes. Different colours represent different anaesthesia levels. For partial TR and TR, only the bars from the control group are visible in both PNDs because the other groups did not show these behaviours. D) The mean ± S.E.M. retrieving time at each test day. The letters in the bar stand for significance. When two bars are indicated with distinct letters the difference between them is significant.

5. Genetic Manipulations

5.1 Mu-Opioid receptor KO pups

The opioid system is involved in mediating processes known to be involved in socially-related distress (Pert & Snyder, 1973). Therefore, a malfunction of this system may hinder dyads' performance in a complex behavioural coordination such as the one required to mothers and pups during a retrieving task. To study the role of pup's opioid system in mother-infant interactions, we produced *Oprm* +/ and -/- pups by crossing *Oprm* +/- mothers with *Oprm* +/- or -/- males (see Kuroda et al., 2011), and the resultant +/ (means +/+ or +/-) and -/- littermate pups were compared for their interaction with biological mothers. mu-Opioid receptors lacking pups' were tested at PNDs 8 and 12. The same animals were tested in the different PNDs. The control pups as well as all the mothers were hetero- or wild-type in respect to the gene encoding mu-opioid receptor.

Results

Maternal behaviours. A significant effect of PND was found on the distribution of maternal position during retrieval, $p < .01$, while no effect of pup genotype was found [Fig.4A, Table 9 of the supplementary materials]. No effect of PND or pup genotype was found on maternal grasp [Fig.4B]. It should be noted that in this mutant mouse line, the rate of atypical maternal

grasp position was consistently very high, irrespective of maternal or pup genotype (see also the next experiment).

Pups' Behaviours. In both groups, TR was only present at PND 12 [Fig.4C]. No effect of PND was found on passive motion, partial TR and struggle. No effect of pup genotype was found on any of the pups' behaviour. Therefore in this study, consistent to our previous results in experimental manual carrying (Yoshida et al., 2013), *Oprm–/–* pups did not show discernible defects in TR during maternal carrying.

Retrieving Time. A main effect of PND was found on retrieving times with longer retrieving times at PND 12 compared to PND 8 [Fig.4D, Table 10 of the supplementary materials]. No significant effect of pup genotype was found on retrieving times.

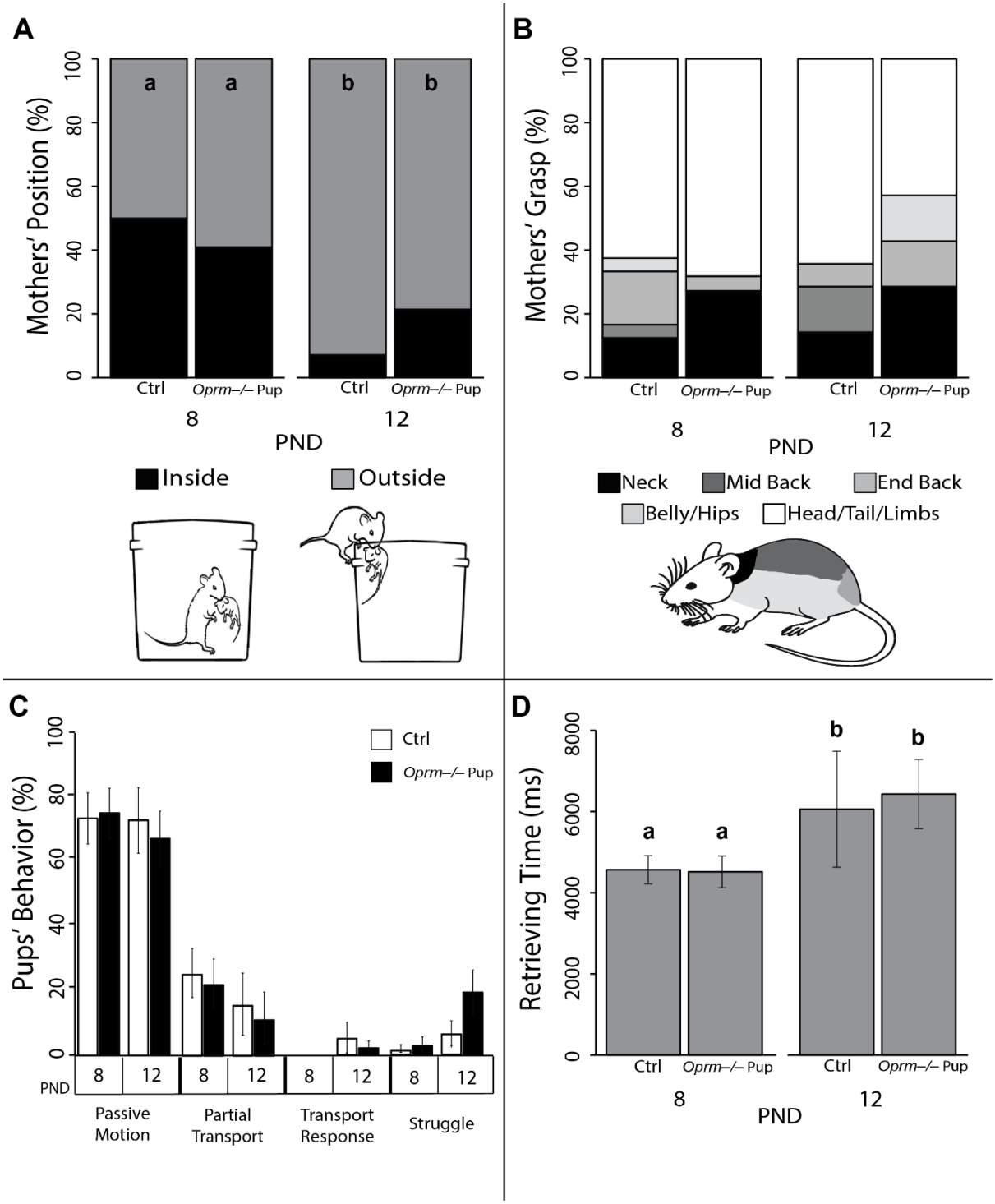


Figure 4. Mothers' and pups' behaviours during the retrieving test in control (N=24 for PND 8, N=14 for PND 12) vs mu-opioid receptors KO pups (N=22 for PND 8, N=14 for PND 12). The same animals were tested in the different PNDs. A) Percentage of retrieving from inside vs outside the cup. B) Percentage of used maternal grasp: neck, mid back, end of back, hips

or belly, and head or tail or limbs. C) Time (% of seconds) spent by pups displaying different behaviours: passive motion, partial TR, TR, and struggle in the two groups: controls (white), and *Oprm-/-* pups (black). D) The mean ± S.E.M. retrieving time at each test day. The letters in the bar stand for significance. When two bars are indicated with distinct letters the difference between them is significant.

5.2 Mu-Opioid receptor KO mothers

An adaptive mutual fit between mothers and pups requires contribution from both parties. Maternal contribution between mothers and pups to the interaction may be challenged by disrupting mothers' own cerebral opioid system. This may be achieved by testing mother-infant dyads in which mothers, instead of pups, are lacking Mu-Opioid receptors. Here mother-infant dyads were tested on PNDs 8 and 12. The same animals were tested in the different PNDs. The control mothers as well as all the pups were hetero- or wild-type with respect to the gene encoding mu-opioid receptor.

Results

Maternal behaviours. A significant effect of PND was found on the distribution of maternal position during retrieval, $p < .05$, while no effect of maternal genotype was found [Fig.5A, Table 11 of the supplementary materials]. No effect of PND or maternal genotype was found on maternal grasp [Fig.5B]. As in Fig. 5b, it should be noted that the rate of atypical maternal grasp position was consistently very high by unknown reason.

Pups' Behaviours. A significant effect of PND but not of maternal genotype was found on TR [Fig.5C, Table 12 of the supplementary materials]. In both groups TR increased on PND 12 compared to PND 8. On the contrary, a significant effect of maternal genotype but not of PND was found on struggle behaviours. At both PNDs, pups struggled more in the group

473 with KO mothers. No effect of PND or maternal genotype was found on passive motion and
474 partial TR.
475 Therefore, even though these *Oprm -/-* mothers themselves showed no apparent
476 behavioural difference from their +/+ and +/ control mothers, the carried pups might have
477 sensed subtle alteration from typical maternal carrying and struggled more.
478
479 *Retrieving Time*. No significant effect of maternal genotype or PND was found on retrieving
480 times [Fig.5d].
481

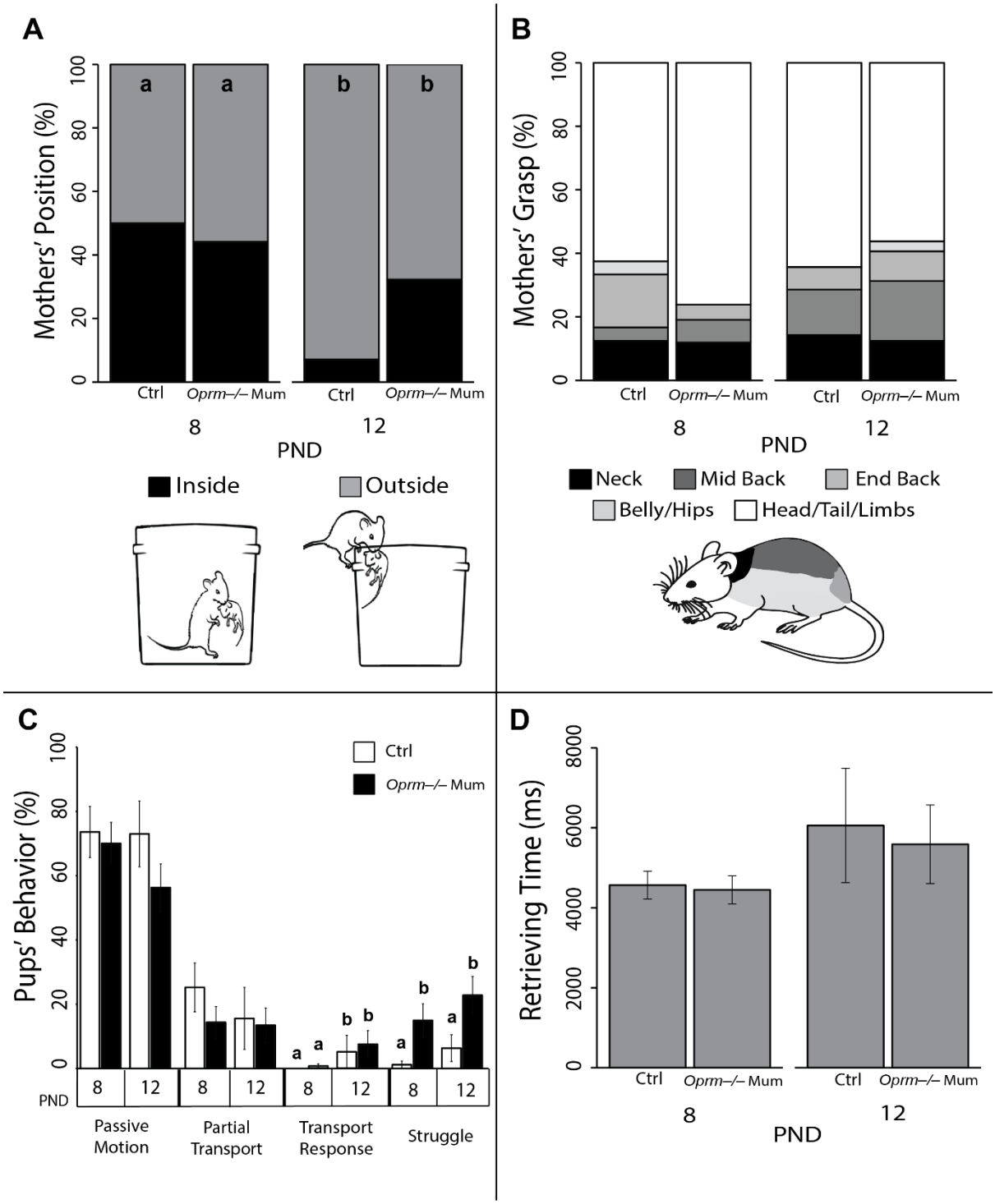


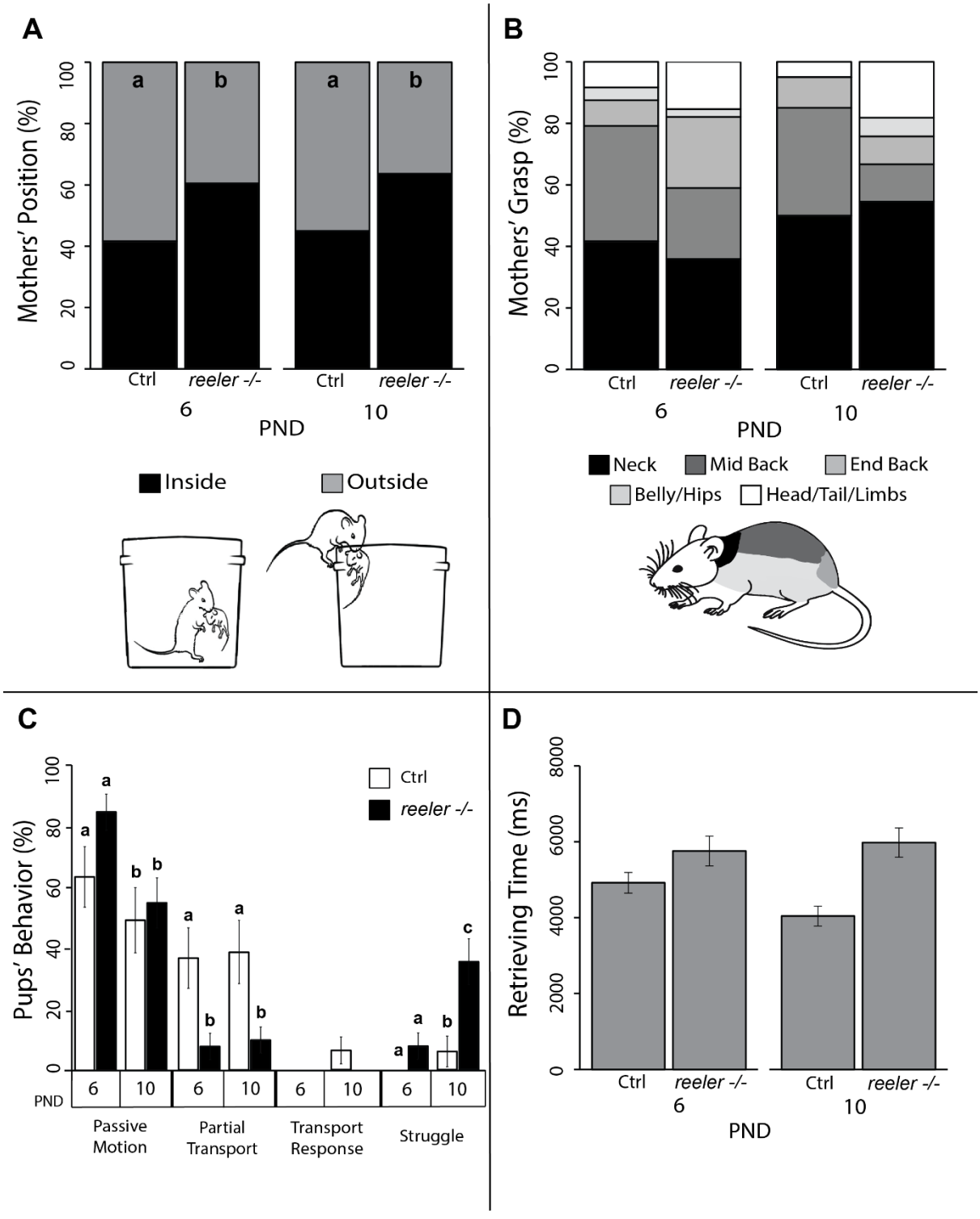
Figure 5. Mothers' and pups' behaviours during the retrieving test in control (N=24 for PND 8, N=14 for PND 12) vs mu-opioid receptors KO mums (N=43 for PND 8, N=32 for PND 12). The same animals were tested in the different PNDs. A) Percentage of retrieving from inside vs outside the cup. B) Percentage of used maternal grasp: neck, mid back, end of back, hips

488 or belly, and head or tail or limbs (for the significance of the multi). C) Time (% of seconds)
489 spent by pups displaying different behaviours: passive motion, partial TR, TR, and struggle
490 in the two groups: controls (white) and *Oprm* -/- mums. D) The mean ± S.E.M. retrieving time
491 at each test day. The letters in the bar stand for significance. When two bars are indicated
492 with distinct letters the difference between them is significant.
493
494 **5.3 *Reeler* mice**
495 Motor coordination is required to obtain an organized and adaptive motor response such as
496 the TR and the proprioceptive input is an essential part of the information necessary for
497 adaptive functioning of the cerebellum – the cerebral structure responsible for motor
498 coordination. To assess whether and to what extent a proper functioning cerebellum is
499 required for pups' adaptive contribution in a retrieving task, genetically mutant pups with ill-
500 developed cerebellum were tested at PNDs 6 and 10. The same animals were tested in the
501 different PNDs. The control pups were all hetero-type in respect to the *reeler* mutation.
502
503 ***Results***
504 *Maternal behaviours*. No significant effect of PND or pup genotype was found on maternal
505 position or grasp during retrieving [Fig.6A-B]. However, a trend was present for maternal
506 position with mothers in the *reeler* -/- group performing a slightly higher percentage of
507 retrieving from inside the cup, p = .051 [Table 13 of the supplementary materials].
508
509 *Pups' Behaviours.* A significant effect of PND but not of pup genotype was found on passive
510 motion [Fig.6C, Table 14 of the supplementary materials]. In both groups, passive motion
511 increased on PND 10 compared to PND 6. On the contrary, a significant effect of pup
512 genotype but not of the PND was found on partial TR, and almost significant interaction
513 between pup genotype and PND was found on struggle were found. At both PNDs, partial
514 TR was higher in the control group compared to the *reeler* -/- group, while at PND 10 only

struggle was higher in the *reeler -/-* group compared to the control group. Full TR was only present in the control group at PND 10.

Retrieving Time. No significant effect of PND or group was found on retrieving times [Fig.6D]. Even though *reeler -/-* pups showed reduced partial TR and increased struggling, the disturbance as shown in the elongation of the retrieval time did not reach statistical significance (see the Discussion).

523



524

525 **Figure 6.** Mothers' and pups' behaviours during the retrieving test in control (N=24 for PND
526 6, N=20 for PND 10) vs homozygous *reeler* mutant pups (N=39 for PND 6, N=33 for PND
527 10). The same animals were tested in the different PNDs. A) Percentage of retrieving from
528 inside vs outside the cup. B) Percentage of used maternal grasp: neck, mid back, end of

529 back, hips or belly, and head or tail or limbs. C) Time (% of seconds) spent by pups
530 displaying different behaviours: passive motion, partial TR, TR, and struggle in the two
531 groups: controls (white) and *reeler -/-* pups. D) The mean ± S.E.M. retrieving time at each
532 test day. The letters in the bar stand for significance. When two bars are indicated with
533 distinct letters the difference between them is significant.
534

535 **6. Tree-based Model**

536 Fig. 7 shows the optimal tree that identifies the relative importance of maternal and pup
537 behavioural parameters, i.e. maternal position, positioning of maternal oral grasp, pup
538 behaviours during carrying, in predicting the retrieval time in all the experiments presented in
539 this paper. The tree model showed that the variable that played the most important role in
540 determining the retrieving time is the maternal position during retrieving. Specifically, when
541 the mother retrieved the infant from the outside of the cup, the rescue was more effective
542 since the retrieving time was shorter. Furthermore, when the mother was retrieving the pups
543 from the outside of the cup, the next most important variable was the position of the maternal
544 grasp. Specifically, retrieving from outside the cup coupled with grasping the pups from the
545 neck or mid back proved to be the most effective combination, irrespective of pups'
546 behaviour. On the other hand, when mothers retrieved the infant from inside the cup, the
547 second moderator of retrieving times was the amount of pups' struggling behaviours. Indeed,
548 when pups struggled for longer than 19% of the time, retrieving times increased, the finding
549 supporting our hypothesis that pup struggling disturbed maternal carrying. Then the third
550 important moderator was the position of maternal grasp. Whether pups struggled or not,
551 maternal neck-grasp was the most preferable for fast retrieving.
552

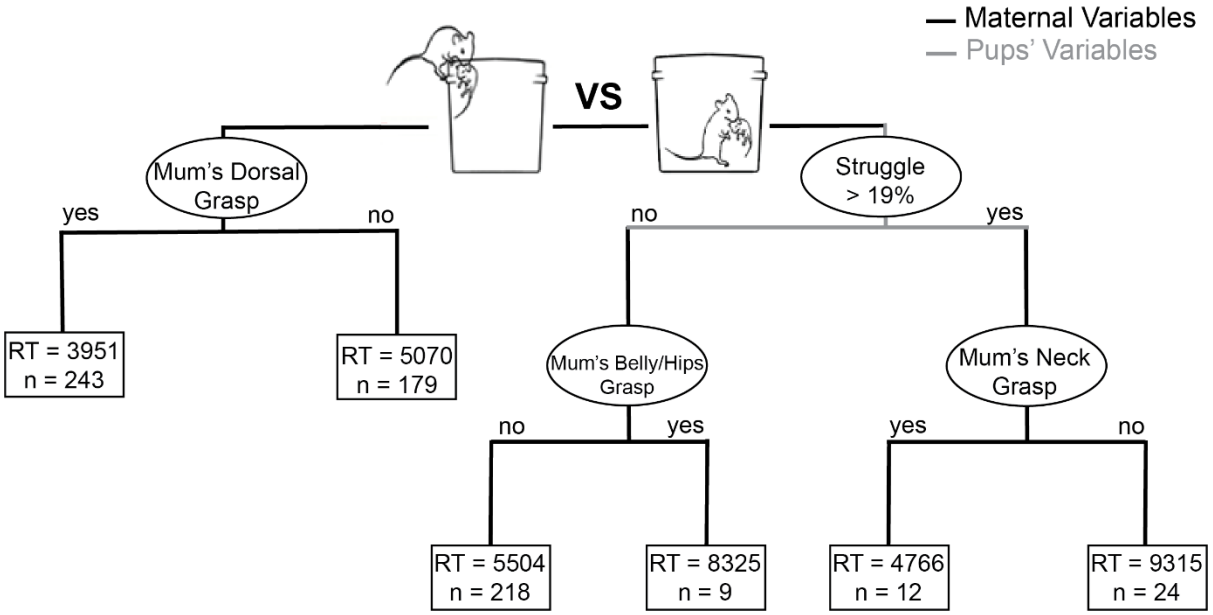


Figure 7. The optimal tree that predicts retrieval time. The regression tree or tree-based model provides information about (1) the hierarchy of the importance of independent variables in explaining the distribution of the dependent variable and (2) which value of the independent variable divides the dependent variable in two groups that differ statistically. The bottom rectangle shows the distribution of retrieving times (RT). The values in the oval leaves of the tree refer to the condition of the independent variable that statistically divides the distribution of the dependent variable. Below each oval leaf, the indications "yes" or "no" refer to whether or not the condition is met. Each leaf is divided in two sub-leaves. The terminal leaves (rectangles) represent subgroups that cannot be further subdivided. The n value in the terminal leaves represents the size of the group.

7. Discussion

In this article, by using the "maternal rescue of pups in a cup" paradigm presented here, we elucidated the development of mother-infant behavioural mutual fit. In typical dyads, the retrieving time decreased along with PNDs. This finding suggests that maternal and pups mutual fit improves with time and experience. The linear model highlighted that the major contribution to the retrieving outcome was given by two pups' behaviours and one maternal

behaviour: combined TR, struggle, and maternal position. As shown previously, pups develop their ability to show full TR in the second postnatal week, and then start struggling more to escape from the cup by themselves after the third postnatal week (Yoshida et al., 2013; Brewster, 1980). On the other hand, along with the postnatal days, mothers constantly increase retrieving from the cup ridge and grasping more and more on the neck at the expense of back, hips, head, tail or limb. Of course, along with the pups' body size increase, the neck becomes higher, and it may become simply easier for the mother to grasp their neck from the top ridge of the cup. Alternatively, the older pups may show more cooperation to maternal oral grasp, so that the mother can get the pup's neck more easily. The orienting behaviour by the infant to maternal holding has been reported in several other mammalian species including infants' anticipatory behaviour for holding (Bowlby, 1969; Shirley, 1933). For example, the lion cubs are often seen to orient their neck by lying down in front of the mother lioness, when they want to be carried (https://www2.edu.ipa.go.jp/%E7%B4%A0%E6%9D%90/%E7%90%86%E7%A7%91/%E7%90%86%E7%A7%91/%E5%8B%95%E7%89%A9/%E3%81%BB%E4%B9%B3%E9%A1%9E%E3%83%8D%E3%82%B3%E7%9B%AE/%E3%83%A9%E3%82%A4%E3%82%AA%E3%83%B3/%E3%83%A9%E3%82%A4%E3%82%AA%E3%83%B3%EF%BC%88%E5%AD%90%E8%82%B2%E3%81%A6%EF%BC%89/). The pharmacological manipulation experiment provides similar implication; when pups were under complete anaesthesia and therefore showed no cooperation to maternal carrying and lying on the floor, the maternal inside position and the retrieval times were increased, compared to those in partial anaesthesia or control groups.

Although originally we tried to investigate the molecular mechanisms of complex mother-infant cooperation, it turned out that none of these genetic mutant models used here could really hinder maternal retrieval efficacy, the most important output measure of this experimental paradigm. This was possibly due to the relative ease of the task for the mother (see below), as well as the capability of both mothers and infants to adjust their behaviour to fit to the atypical behaviour in the counterpart. For example, *reeler -/-* pups showed altered

behavioural response during carrying, including the elongated body length, head direction, hindlimb and tail positioning (Esposito et al., 2013), the maternal adjustment of positioning more from inside for *reeler -/-* pups might be effective enough to maintain the swift retrieval. It should also be noted that both *reeler -/-* (Esposito 2013) and *Oprm -/-* (Yoshida 2013) pups show normal immobilization responses.

The final analysis using the tree-based model, taking into account the entire dataset, highlighted that the variable that best predicts the outcome in terms of retrieving times is the maternal position. Specifically, when mothers retrieve from the cup ridge, retrieving times are lower, irrespective of the pup's behaviour, suggesting that this is the easiest way of pup rescue in this task. However, when the mother needs to come inside the cup to grasp the pup, the pup's behavioural contribution become visible. This fact also tells the limitation of this current version of "maternal rescue of pups in a cup" assay that it is maybe too easy if the mother can grasp a pup from the top ridge, so that the retrieval time did not elongated by pups' postural alteration (as seen in *reeler -/-* pups) or even much more struggling (Fig. 3D). When desirable, therefore, one should modify the experimental setting in a way to adjust the task ease be more suitable to detect the phenomenon of interest: for example, to change the depth or shape of the cup as described in the Method section 2.2 will easily alter the ease of the task for the mother. If one wish to make the task more demanding for the mother, the maternal retrieval path can be elongated and include barriers, such as a narrow and long tunnel as performed in a previous study (Brewster, 1980). In this case, however, the experiments will not be able to be done in the standard animal husbandry, and will need a separate testing room. The benefit of the present "maternal rescue of pups in a cup" task is that it is compatible in a shoe-box sized ventilated cage often used in Specific-Pathogen-Free animal facility. The simple, low-cost, and naturalistic setting may be an interesting addition of behavioural screening assays for mother-infant interactions of genetic mutant mouse strains. The lack of behavioral effects in the *reeler* mutant could have been the consequence of the fact that backcrossing was not applied, rather than the result of the easiness of the task. Indeed, Mutant mice and wild-type littermates have more alleles of ES

628 cells (129S in this case) and of a breeder line (C57BL/6J), respectively, near the deleted
629 gene. Thus, the effects, or lack therefor, of reeler KO on behaviors might not be due to
630 reeler deletion, but to 129S alleles alone or combination of 129S alleles and reeler deletion.
631 In future studies backcrossing should be applied in order to disentagle between these two
632 possibilities.
633
634 **8. Conclusions**
635 It has been shown that mother-infant mutual fit is a complex construct and that several
636 intermingled mechanisms are involved in the development of real-life mother-infant
637 interactions. Mothers and infants, when interacting, should be considered together as one
638 whole system in which any change in one side or the other, affects the output of the whole
639 dyad. The outcome of the interaction relays on a specific dynamic pattern of infant and
640 maternal behaviours which mutually change and adapt to fit each other's needs. Key
641 features to reach a successful outcome of the interaction were the maternal retrieving
642 strategy and infants' TR behaviour. This study aims to deepen insight on the underlying
643 physiological and genetic mechanisms of infant and maternal interactions by applying the
644 retrieving paradigm together with pharmacological and genetic manipulations. Specifically,
645 the lower level sensory-motor control seems to be more involved in this specific task
646 compared to higher cerebral processes. Indeed, lack of proprioceptive and tactile input
647 reduced or hindered TR elicitation (Esposito et al, 2013). For finer, more sophisticated
648 adaptations to the social context such as a short period of maternal absence from the cage,
649 we have recently generated another behavioural paradigm, and showed its mediation
650 through the anterior cingulate cortex (Yoshida et al., 2013). In this way, the behavioural
651 paradigms involving the TR would be a new repertoire to investigate the core behavioural
652 features of mother-infant interactions in several mammalian species. Results of this study
653 found that the variable that best predicts retrieval time is maternal position, and position of
654 maternal grasp from outside the cup. If retrieval was carried out by the mother within the
655 cup, the pups' behaviour then becomes a critical variable that predicts retrieval time. This

study also showed that mothers possess high capability to adjust their behaviour to fit best to the condition of pups, so that the retrieval time was often not affected by the disturbance of pups' TR. To detect the alteration of mother-pup dyad more sensitively, the relative demandingness of behavioural paradigm should be modified by customisation of the experimental settings.

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Maternal rescue of pups in a cup: A naturalistic behavioral assay to investigate mother-infant interactions in mice/rodents

Supplementary Materials

Here the results from the logistic and linear models are reported.

1. Typical Development

Maternal Position. As reported in the main text, the logistic model showed a significant effect of PND on maternal positioning during successful retrieval, $p < .01$ [Table 1]. Specifically, maternal retrieving from the cup ridge increased steadily in subsequent post-natal days, while retrieving from inside decreased steadily.

	Odds ratio	95%CI low	95%CI high	*z* value	*p* value	
(Intercept)	2.142	0.846	5.562	1.596	0.111	
PND	0.864	0.783	0.946	-3.049	0.002	**

Table 1. Results from the binary logistic regression run on maternal position in the typical development group. Initial model: ~ PND (AIC = 145.39). Best fitted model: ~ PND. *p<.05, **p<.01, ***p<.001

Maternal Grasp. A significant effect of PND was found on the distribution of pups' body parts grasped by the mother [Table 2]. Grasping from the neck became more frequent in subsequent post-natal days compared to the use of mid back, end back, or head/tail/limbs grasps which decreased steadily over time (Mid back: $p < .05$; End back: $p < .05$; Head/tail/limbs: $p < .001$). No significant effect of PND was found on neck vs belly/hips grasps.

"Middle of backbone" vs "neck"

	Odds ratio	95%CI low	95%CI high	*z* value	*p* value	
(Intercept)	1.607	0.442	5.842	0.720	0.471	
PND	0.889	0.791	0.999	-1.984	0.047	*

"End of backbone" vs "neck"

	Odds ratio	95%CI low	95%CI high	*z* value	*p* value	
(Intercept)	1.363	0.297	6.254	0.399	0.690	
PND	0.846	0.728	0.982	-2.196	0.028	*

"Hips or belly" vs "neck"

	Odds ratio	95%CI low	95%CI high	*z* value	*p* value	
(Intercept)	64.031	0.000	7.17E+14	0.271	0.786	
PND	0.223	0.000	386.708	-0.395	0.693	

"Head, limbs or tail" vs "neck"

	Odds ratio	95%CI low	95%CI high	*z* value	*p* value	
(Intercept)	4.827	1.419	16.424	2.520	0.012	*
PND	0.792	0.699	0.897	-3.671	0.000	***

Table 2. Results from the multinomial logistic regression run on maternal grasp in the typical development group. Initial model: ~ PND (AIC = 301.76). Best fitted model: ~ PND. *p<.05, **p<.01, ***p<.001

Pups' Behaviors. PND has a significant effect on the distribution of passive motion, partial transport and transport response behaviors [Table 3]. Specifically, PND had a significant effect with passive motion decreasing steadily over time and partial transport and transport response increasing steadily over time (Passive motion: $p < .001$; Partial TR: $p < .001$; TR: $p < .001$). On the contrary, PND showed no significant effect on struggle behavior.

a) "Passive Motion"

	Coefficient	SE	*t* value	*p* value	
(Intercept)	1.162	0.079	14.746	0.000	***
PND	-0.061	0.007	-8.371	0.000	***

b) "Partial Transport"

	Coefficient	SE	*t* value	*p* value	
(Intercept)	-0.037	0.065	-0.570	0.570	
PND	0.025	0.006	4.181	0.000	***

c) "Transport Response"

	Coefficient	SE	*t* value	*p* value	
(Intercept)	-0.138	0.052	-2.654	0.009	**
PND	0.029	0.005	6.044	0.000	***

d) "Struggle"

	Coefficient	SE	*t* value	*p* value
(Intercept)	0.013	0.052	0.255	0.799
PND	0.007	0.005	1.438	0.153

Table 3. Results from the linear regressions run on pups' behaviors in the typical development group. a) Passive Motion: Initial model: ~ PND (AIC = -244.2). Best fitted model: ~ PND b) Partial Transport: Initial model: ~ PND (AIC = -290.24). Best fitted model: ~ PND. c) Transport Response: Initial model: ~ PND (AIC = -341.31). Best fitted model: ~ PND. d) Struggle: Initial model: ~ PND (AIC = -342.41). Best fitted model: ~ PND. *p<.05, **p<.01, ***p<.001

Retrieving Time. A significant main effect of age was found on retrieval time, p < .01 [Table 4].

	Coefficient	SE	*t* value	*p* value	
(Intercept)	7057.330	675.300	10.451	0.000	***
PND	-187.560	62.560	-2.998	0.003	**

Table 4. Results from the linear regression run on retrieving time in the typical development group. Initial model: ~ PND (AIC = 1874.95). Best fitted model: ~ PND. *p<.05, **p<.01, ***p<.001

2. Pharmacological Manipulations

Maternal Position. A significant effect of anesthesia was found in the complete anesthesia (A40) group on the distribution of maternal position, p < .01 [Table 5]. Mothers in the A40 group showed an overall increase of retrieval from inside the cup compared to the mothers in the control group.

	Odds ratio	95%CI low	95%CI high	*z* value	*p* value	
(Intercept)	0.305	0.175	0.506	-4.409	0.000	***
A30	1.229	0.470	3.072	0.435	0.664	
A40	4.097	1.783	9.696	3.279	0.001	**

Table 5. Results from the binary logistic regression run on maternal position in control vs anesthetized pups (A30: 30mg/kg; A40: 40mg/kg). Initial model: ~ PND + anesthesia level +

interaction (AIC = 181.72). Best fitted model: ~ anesthesia level (AIC = 177.88). *p<.05, **p<.01, ***p<.001

Maternal Grasp. Mothers in the partial anesthesia group increased the use of head/tail/limbs grasping across PNDs compared to control group, $p < .05$ [Table 6].

"Middle of backbone" vs "neck"

	Odds ratio	95%CI low	95%CI high	*z* value	*p* value	
(Intercept)	0.595	0.351	1.008	-1.931	0.054	.
A30	0.917	0.297	2.828	-0.151	0.880	
A40	0.600	0.190	1.895	-0.870	0.384	

"End of backbone" vs "neck"

	Odds ratio	95%CI low	95%CI high	*z* value	*p* value	
(Intercept)	0.135	0.053	0.344	-4.200	0.000	***
A30	0.672	0.071	6.379	-0.346	0.729	
A40	0.528	0.057	4.930	-0.561	0.575	

"Hips or belly" vs "neck"

	Odds ratio	95%CI low	95%CI high	*z* value	*p* value	
(Intercept)	0.000	0.000	1.06E+106	-0.102	0.919	
A30	57964.730	0.000	3.97E+116	0.083	0.933	
A40	228052.000	0.000	1.55E+117	0.094	0.925	

"Head, limbs or tail" vs "neck"

	Odds ratio	95%CI low	95%CI high	*z* value	*p* value	
(Intercept)	0.351	0.187	0.661	-3.244	0.001	**
A30	3.621	1.317	9.956	2.494	0.013	*
A40	2.439	0.900	6.609	1.753	0.080	.

Table 6. Results from the multinomial logistic regression run on maternal grasp in control vs anesthetized pups (A30: 30mg/kg; A40: 40mg/kg). Initial model: ~ PND + anesthesia level + interaction (AIC = 395.04). Best fitted model: ~ anesthesia level (AIC = 391.06). *p<.05, **p<.01, ***p<.001

Pups' Behaviors. Analysis showed significant effects of PND and anesthesia group on pup's behaviors [Table 7]. At PND 8 pups in the partial anesthesia group showed more struggle and less passive motion compared to control pups, while the pups in the complete

anesthesia group showed almost always passive motion. Partial TR and TR only occurred in the control group and, within the control group, TR increased in PND 12 compared to PND 8.

"Passive Motion"

	Coefficient	SE	*t* value	*p* value	
(Intercept)	1.455	0.214	6.801	0	***
PND	-0.093	0.021	-4.399	0	***
A30	-0.846	0.392	-2.158	0.033	*
A40	-0.349	0.38	-0.921	0.359	
PND:A30	0.061	0.039	1.587	0.115	
PND:A40	0.08	0.037	2.156	0.033	*

"Passive Motion": PND 8 only

	Coefficient	SE	*t* value	*p* value	
(Intercept)	0.707	0.06	11.742	0	***
A30	-0.354	0.111	-3.184	0.002	**
A40	0.293	0.109	2.686	0.009	**

"Passive Motion": PND 12 only

	Coefficient	SE	*t* value	*p* value	
(Intercept)	0.333	0.059	5.606	0	***
A30	-0.108	0.107	-1.011	0.316	
A40	0.614	0.101	6.069	0	***

"Partial Transport"

Null model

"Transport Response"

	Coefficient	SE	*t* value	*p* value	
(Intercept)	-0.289	0.13	-2.232	0.029	*
PND	0.039	0.013	3.028	0.003	**

"Struggle"

	Coefficient	SE	*t* value	*p* value	
(Intercept)	-0.179	0.109	-1.641	0.103	
PND	0.024	0.011	2.233	0.027	*
A30	0.652	0.054	12.173	0	***
A40	-0.033	0.052	-0.638	0.525	

Table 7. Results from the linear regressions run on pups' behaviors in control vs anesthetized pups (A30: 30mg/kg; A40: 40mg/kg). a) Passive Motion: Initial model: ~ PND + anesthesia level + interaction (AIC = -284.85). Best fitted model: ~ PND + anesthesia level + interaction. b) Passive Motion at PND 8: Initial model: ~ anesthesia level (AIC = -141.90).

Best fitted model: ~ anesthesia level. c) Passive Motion at PND 12: Initial model: ~ anesthesia level (AIC = -143.41). Best fitted model: ~ anesthesia level. d) Partial Transport, present in the control group only: Initial model: ~ PND (AIC = -136.5). Best fitted model: *null model* (AIC = -136.9). e) Transport Response, present in the control group only: Initial model: ~ PND (AIC = -227.46). Best fitted model: ~ PND. f) Struggle: Initial model: ~ PND + anesthesia level + interaction (AIC = -391.48). Best fitted model: ~ PND + anesthesia level (AIC = -395.08). *p<.05, **p<.01, ***p<.001

Retrieving Time. A significant main effect of the anesthesia group was found on retrieval time, p<.001) [Table 8]. Specifically, in the group where pups had received the complete anesthesia (40mg/kg) retrieving times were higher compared to the control group at both PNDs.

	Coefficient	SE	*t* value	*p* value	
(Intercept)	5299.950	870.230	6.090	0.000	***
PND	-140.300	84.910	-1.652	0.101	
A30	840.150	427.880	1.964	0.052	.
A40	2159.240	411.630	5.246	0.000	***

Table 8. Results from the linear regression run on retrieving time in control vs anesthetized pups (A30: 30mg/kg; A40: 40mg/kg). Initial model: ~ PND + anesthesia level + interaction (AIC = 2247.99). Best fitted model: ~ PND + anesthesia level (AIC = 2246.77). *p<.05, **p<.01, ***p<.001

3. Genetic Manipulations

3.1 Mu-Opioid receptor KO pups

Maternal Position. A significant effect of PND was found on the distribution of maternal position during retrieval, p < .01, while no effect of pup genotype was found [Table 9].

	Odds ratio	95%CI low	95%CI high	*z* value	*p* value	
(Intercept)	21.337	1.546	406.372	2.189	0.029	*

PND	0.667	0.478	0.885	-2.626	0.009	**

Table 9. Results from the binary logistic regression run on maternal position in control vs Mu-opioid receptor KO pups. Initial model: ~ PND + genotype + interaction (AIC = 92.79). Best fitted model: ~ PND (AIC = 90.39). *p<.05, **p<.01, ***p<.001

Maternal Grasp. No effect of PND or pup genotype was found on maternal grasp (Initial model: ~ PND + genotype + interaction, AIC = 183.47; Best fitted model: *null model*, AIC = 174.71).

Pup's Behaviors. No effect of PND was found on passive motion (Initial model: ~ PND + genotype + interaction, AIC = -142.12; Best fitted model: *null model*, AIC = -147.69), partial TR (Initial model: ~ PND + genotype + interaction, AIC = -147.36; Best fitted model: *null model*, AIC = -151.65), and struggle [Table 10]. No effect of pup genotype was found on any of the pups' behavior.

"Passive Motion"
Null model

"Partial Transport"
Null model

"Transport Response"
Null model

"Struggle"

	Coefficient	SE	*t* value	*p* value
(Intercept)	-0.091	0.121	-0.751	0.455
PND	0.013	0.012	1.029	0.307
KO	-0.213	0.174	-1.226	0.225
PND:KO	0.029	0.018	1.595	0.115

Table 10. Results from the linear regressions run on pups' struggle behavior in control vs Mu-opioid receptor KO pups. Initial model: ~ PND + genotype + interaction (AIC = -271.83). Best fitted model: ~ PND + genotype + interaction.

Retrieving Time. A main effect of PND was found on retrieving times with longer retrieving times at PND 12 compared to PND 8 [Table 11]. No significant effect of pup genotype was found on retrieving times.

	Coefficient	SE	*t* value	*p* value	
(Intercept)	1136.800	1737.200	0.654	0.515	
PND	425.700	178.900	2.379	0.020	*

Table 11. Results from the linear regression run on retrieving time in control vs Mu-opioid receptor KO pups. Initial model: ~ PND + genotype + interaction (AIC = 1190.1). Best fitted model: ~ PND (AIC = 1186.22). *p<.05, **p<.01, ***p<.001

3.1 Mu-Opioid receptor KO mums

Maternal Position. A significant effect of PND was found on the distribution of maternal position during retrieval, $p < .05$, while no effect of maternal genotype was found [Table 12].

	Odds ratio	95%CI low	95%CI high	*z* value	*p* value	
(Intercept)	169.000	2.702	79241.870	2.129	0.033	*
PND	0.527	0.250	0.833	-2.300	0.021	*
KO	0.013	0.000	1.591	-1.615	0.106	
PND:KO	1.672	0.988	3.620	1.687	0.092	.

Table 12. Results from the binary logistic regression run on maternal position in control vs Mu-opioid receptor KO mums. Initial model: ~ PND + genotype + interaction (AIC = 146.49). Best fitted model: ~ PND + genotype + interaction. *p<.05, **p<.01, ***p<.001

Maternal Grasp. No effect of PND or maternal genotype was found on maternal grasp (Initial model: ~ PND + genotype + interaction, AIC = 259.69; Best fitted model: *null model*, AIC = 245.59).

Pups' Behaviors. A significant effect of PND but not of maternal genotype was found on TR [Table 13]. On the contrary, a significant effect of maternal genotype but not of PND was found on struggle behaviors. At both PNDs, pups struggled more in the group with KO mothers. No effect of PND or maternal genotype was found on passive motion and partial TR.

"Passive Motion"
Null model

"Partial Transport"
Null model

"Transport Response"

	Coefficient	SE	*t* value	*p* value	
(Intercept)	-0.121	0.069	-1.763	0.081	.
PND	0.016	0.007	2.246	0.027	*

"Struggle"

	Coefficient	SE	*t* value	*p* value	
(Intercept)	0.031	0.046	0.682	0.497	
KO	0.151	0.056	2.696	0.008	**

Table 13. Results from the linear regressions run on pups' behaviors in control vs Mu-opioid receptor KO mums. a) Passive Motion: Initial model: ~ PND + genotype + interaction (AIC = -194.7). Best fitted model: *null model* (AIC = -197.32). b) Partial Transport: Initial model: ~ PND + genotype + interaction (AIC = -240.8). Best fitted model: *null model* (AIC = -244.67). c) Transport Response: Initial model: ~ PND + genotype + interaction (AIC = -420.46). Best fitted model: ~ PND (AIC = -424.17). d) Struggle: Initial model: ~ PND + genotype + interaction (AIC = -277.05). Best fitted model: ~ genotype (AIC = -279.29). *p<.05, **p<.01, ***p<.001

Retrieving Time. No significant effect of maternal genotype or PND was found on retrieving times [Table 14].

	Coefficient	SE	*t* value	*p* value
(Intercept)	2012.000	1798.000	1.119	0.266
PND	310.000	183.000	1.694	0.093

Table 14. Results from the linear regression run on retrieving time in control vs Mu-opioid receptor KO mums. Initial model: ~ PND + genotype + interaction (AIC = 1869.51). Best fitted model: ~ PND (AIC = 1866.19).

5.3 *Reeler* mice

Maternal position. A trend was present for maternal position with mothers in the *reeler* -/- group performing a slightly higher percentage of retrieving from inside the cup, $p = .051$ [Table 15].

	Odds ratio	95%CI low	95%CI high	*z* value	*p* value
(Intercept)	0.760	0.413	1.375	-0.902	0.367
KO	2.144	1.004	4.661	1.954	0.051

Table 15. Results from the binary logistic regression run on maternal position in control vs *reeler* -/- pups. Initial model: ~ PND + genotype + interaction (AIC = 162.37). Best fitted model: ~ genotype (AIC = 158.49). *p<.05, **p<.01, ***p<.001

Maternal Grasp. No effect of PND or maternal genotype was found on maternal grasp (Initial model: ~ PND + genotype + interaction, AIC = 333.19; Best fitted model: *null model*, AIC = 323.55).

Pups' Behaviors. A significant effect of PND but not of pup genotype was found on passive motion [Table 16]. In both groups passive motion increased on PND 10 compared to PND 6. On the contrary, a significant effect of pup genotype but not of the PND was found on partial TR and struggle. At both PNDs partial TR was higher in the control group compared to the *reeler* -/- group, while at PND 10 only struggle was higher in the *reeler* -/- group compared to the control group. Full TR was only present in the control group at PND 10.

"Passive Motion"

	Coefficient	SE	*t* value	*p* value	
(Intercept)	1.035	0.174	5.941	0	***
PND	-0.06	0.021	-2.893	0.005	**
KO	0.142	0.085	1.675	0.097	.

"Partial Transport"

	Coefficient	SE	*t* value	*p* value	
(Intercept)	0.375	0.053	7.061	0	***
KO	-0.289	0.067	-4.281	0	***

"Transport Response"
Only present in control pups at PND 10

"Struggle"

	Coefficient	SE	*t* value	*p* value
(Intercept)	-0.089	0.179	-0.5	0.618
PND	0.015	0.022	0.673	0.502
KO	-0.248	0.227	-1.095	0.276
PND:KO	0.054	0.028	1.929	0.056

"Struggle: PND 6 only"
Null model

"Struggle: PND 10 only"

	Coefficient	SE	*t* value	*p* value	
(Intercept)	0.06	0.082	0.729	0.469	
KO	0.294	0.104	2.836	0.007	**

Table 16. Results from the linear regressions run on pups' behaviors in control vs *reeler* -/- pups. a) Passive Motion: Initial model: ~ PND + genotype + interaction (AIC = -185.42). Best fitted model: ~ PND + genotype (AIC = -186.56). b) Partial Transport: Initial model: ~ PND + genotype + interaction (AIC = -236.18). Best fitted model: ~ genotype (AIC = -240.09). c) Struggle: Initial model: ~ PND + genotype + interaction (AIC = -281.24). Best fitted model: ~ PND + genotype + interaction. d) Struggle at PND 6: Initial model: ~ genotype (AIC = -192.85). Best fitted model: *null model* (AIC = -192.88). e) Struggle at PND 10: Initial model: ~ genotype (AIC = -104.71). Best fitted model: ~ genotype. *p<.05, **p<.01, ***p<.001

Retrieving Time. No significant effect of pup genotype or PND was found on retrieving times [Table 17].

	Coefficient	SE	*t* value	*p* value	
(Intercept)	6232.200	1230.200	5.066	0.000	***
PND	-219.100	152.500	-1.437	0.154	
KO	-807.200	1562.200	-0.517	0.606	
PND:KO	274.300	193.500	1.418	0.159	

Table 17. Results from the linear regression run on retrieving time in control vs *reeler* -/- pups. Initial model: ~ PND + genotype + interaction (AIC = 1769.02). Best fitted model: ~ PND + genotype + interaction.