

Repeated Cross-fostering Protocol as a Mouse Model of Early Environmental Instability

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[Abstract] Early life events have a crucial role in programming the individual phenotype indeed the exposure to traumatic experiences during infancy can increase later risks for a variety of neuropsychiatric conditions, including mood and anxiety disorders. Several studies in rodents demonstrated the impact of short and long sessions of separation/isolation from caregivers in developing pups, on the behavioral and hormonal response to stress during infancy and adulthood (D'Amato *et al.*, 1998; Meaney *et al.*, 2000; Luchetti *et al.*, 2015). The repeated cross-fostering (RCF) is an early manipulation carried out in mouse pups during the first four postnatal days life. Differently from other early manipulations, hypothalamic-pituitary-adrenal (HPA) axis functioning is not altered in RCF treated subjects. This manipulation is used to model human early environmental instability, a risk factor for internalizing disorders including separation anxiety disorder, panic disorder and CO₂ hypersensitivity (Kendler *et al.*, 1992; Forman and Davies, 2003; Battaglia *et al.*, 2009).

Materials and Reagents

Note: See Figure 1.

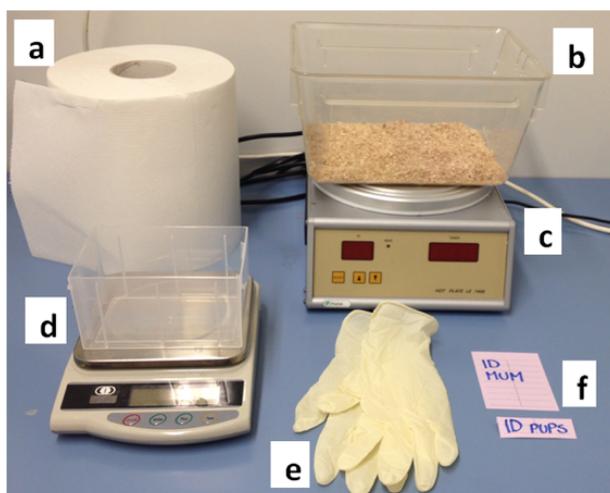


Figure 1. Materials and equipment. a. paper towels for cage enrichment; b. clean cage; c. hot plate; d. balance; e. latex gloves; f. tags for mum and pups identification.

1. Paper towels for cage enrichment
2. Gloves to manipulate animals (latex)
3. Balance
4. 10 females (minimum number) and 5 males of the same mouse strain (we use NMRI, C57BL6 and DBA, but it is possible to use this protocol in other strains) for mating

Equipment

Note: See Figure 1.

1. Clean cages to isolate pregnant females and to temporarily maintain pups on the hot plate during the manipulation, as described at point 2 of "procedure step by step" (washed transparent high temperature polysulfone cages 26.7 x 20.7 x 14.0 cm, with clean bedding)

Procedure

1. Mating procedure consists of housing 2 females with one male for 15 d. The experimental procedure requires that, at least 5 females give birth simultaneously, within 24 h. To reach this goal and synchronize females' estrous cycles, females can be exposed to male odor a few days before mating. Check female weight at mating.
2. On the 15th day, males are removed and females are weighed to assess pregnant status. The weight gained, suggesting pregnancy, depends on the strain of mice used, but generally is between 10 and 15 grams in 15 days (a 50% increase of weight from mating day); in addition visible lateral "bulges" are visible in pregnant females.
3. Each pregnant female is isolated in a clean cage with bedding material (paper) and daily checked for pups' birth (PND0: postnatal day 0).
4. At least 5 females should give birth within 24 h to permit the execution of RCF manipulation.

Rcf manipulation

Pups spend the first postnatal day (PND0) with the biological mother. Differently from the "classical" cross-fostering procedures (Bartolomucci *et al.*, 2004), RCF pups change caregiver every 24 h for 4 times during the PND1-PND4 time interval, by following a rotation scheme: Each dam receives 4 different litters during this manipulation and each litter changes 4 different adoptive dams (the adoptive dam is the mum who receive pups from another mum) (also see Figure 2 and the Table 1). The daily procedure consists of first removing the mother from the cage, then removing its entire litter, and immediately introducing this litter into the home-cage of a different dam whose pups had just been removed. The RCF pups were then semi-covered with the home-cage bedding of the adoptive mother, which is then reintroduced in the cage and left with this litter for the next

24 h. The entire procedure lasted about 30 sec and took place every day at same time during the light cycle of a standard light/dark cycle (usually between 10.30 and 11.00 a.m.). This was repeated daily, four times until reaching the fourth adoptive mother, with which pups were left until weaning (PND 28).

Adoptive dams should be lactating females with pups of the same age as fostered litters and litters should have same or at least similar number of pups to avoid possible nutritional stress for mothers and pups.

The control manipulation consists in first removing the mother from the cage, then picking up control pups, reintroducing them in their home-cage and covering with home-cage bedding. At the end of this manipulation, which lasts 30 sec, the biological mother is reintroduced in the home cage. This control procedure takes place daily from PND1 to PND4, to assess possible effects of the handling procedure necessarily required by RCF protocol. It is not necessary that control females give birth exactly on the same day.

Procedure step by step

For example, we consider 5 litters RCF and one CONTROL. We identify RCF mums with uppercase letters A, B, C, D and E, and respective pups with the same but lowercase letters a, b, c, d, and e. The control mum identification is F and respective pups f.

To facilitate the procedure we record mum's code on the home cage tag while the pups' code is written on a smaller removable tag that can be transferred (together with pups) to the adoptive mother's home-cage tag.

First day (PND1)

1. Remove mum "A" from its home cage.
2. Remove pups "a" from the home cage and put them in a clean cage on hot plate (30-35 °C) to maintain body temperature.
3. Remove mum "B" from its home cage.
4. Move pups "b" from their home cage to the mum's "A" cage, semi-cover pups with bedding and reintroduce mum "A" in its home cage.
5. Remove mum "C" from its home cage.
6. Move pups "c" from their home cage to the mum's "B" cage, cover pups with some bedding and reintroduce mum "B" in its home cage.
7. Remove mum "D" from its home cage.
8. Move pups "d" from their home cage to the mum's "C" cage, cover pups with some bedding and reintroduce mum "C" in its home cage.
9. Remove mum "E" from its home cage.
10. Move pups "e" from their home cage to the mum's "D" cage, cover pups with some bedding and reintroduce mum "D" in its home cage.
11. Put pups "a" in the mum's "E" cage, cover pups with some bedding and reintroduce mum "E" in its home cage.

The following days (PND 2,3,4) the procedure always starts from the mum "A".

The control manipulation consists in first removing the mother “F” from the cage, than picking up pups “f” and reintroducing them in their home-cage and covering with home-cage bedding. At the end of this manipulation, which lasts 30 sec, the biological mother “F” is reintroduced in the home cage. This manipulation is repeated for the PND1, PND2, PND3 and PND4.

Home cage bedding was changed on postnatal day 10.

Representative data

A general overview of behavioral and physiological outcomes of the RCF protocol in NMRI mice is reported in Luchetti *et al.* (2015).

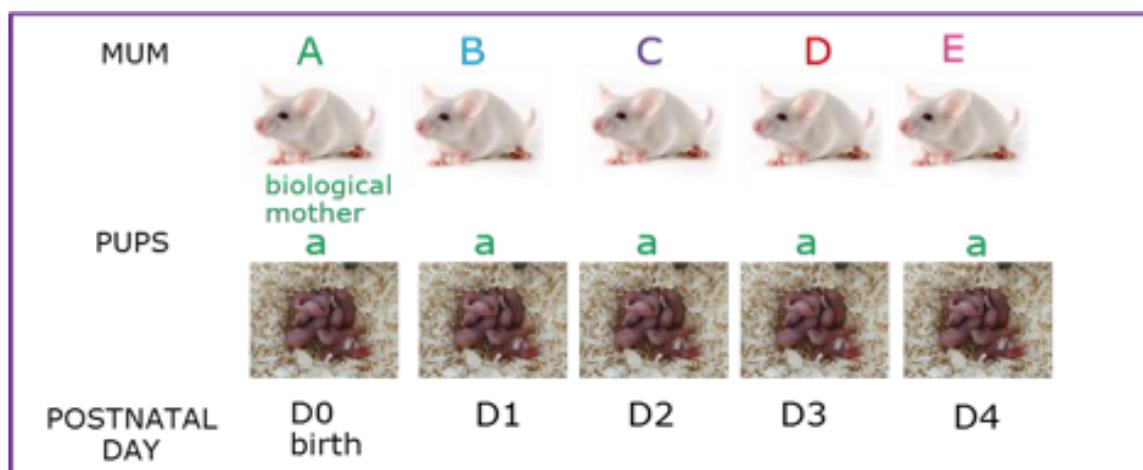


Figure 2. Schematic representation of RCF procedure. Pups "a" born from mum "A" spend the first day (D0) with their mum. Then they change caregiver for four consecutive days spending D1 with mum "B", day 2 with dam "C", day D3 with mum "D" and finally from D4 to weaning with adoptive mum "E".

Table 1. Rotation scheme of pups during the repeated cross fostering manipulation.

(PND = post-natal day)

MOTHERS	PUPS				
	PND 0 (birth)	PND 1	PND 2	PND 3	PND 4
A	a	b	c	d	e
B	b	c	d	e	a
C	c	d	e	a	b
D	d	e	a	b	c
E	e	a	b	c	d

Troubleshooting

In some cases (rarely) one/two pups could die/disappear during the days of manipulation and in these cases we usually continue the RCF protocol. To promote adoption, we introduced pups to be adopted in the already present nest of the adoptive dam, and scattered a few amount of her home-cage bedding (and nest material) over them. Following this procedure we never observed pups' rejection.

We suggest not using the same females for more than one experiment.

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