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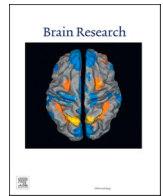
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## Research paper

# Early movement restriction affects the acquisition of neurodevelopmental reflexes in rat pups

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## ABSTRACT

Childhood is a period of construction of the organism, during which interactions with the environment and regular physical activity are necessary for the maturation of the neuronal networks. An atypical sensorimotor activity during childhood (due to bed-rest or neurodevelopmental disorders) impacts the development of the neuromuscular system. A model of sensorimotor restriction (SMR) developed in rats has shown that casting pups' hind limbs from postnatal day 1 (P1) to P28 induced a severe perturbation of motor behavior, due to muscle weakness as well as disturbances within the central nervous system. In the present study, our objective was to determine whether SMR affects the early postnatal ontogenesis. We explored the neuromuscular development through the determination of the age for achievement of the main neurodevelopmental reflexes, which represent reliable indicators of neurological and behavioral development. We also evaluated the maturation of postural control. Our results demonstrate that SMR induces a delay in the motor development, illustrated by a several days delay in the acquisition of a mature posture and in the acquisition reflexes: hind limb grasping, righting, hind limb placing, cliff avoidance, negative geotaxis. In conclusion, impaired physical activity and low interactions with environment during early development result in altered maturation of the nervous system.

## 1. Introduction

Childhood is a period of organism construction, subject to environmental influences, and it is now well established that physical activity, interactions with the environment and social interactions are necessary for a typical and harmonious maturation of the organization and functions of the central nervous system (Luhmann et al., 2016). Being physically active early in life has structural consequences on the brain (Valkenborghs et al., 2019), reduces the risk of dementia and preserves cognitive functions later in life (Hotting and Roder, 2013). Therefore, the durations of physical activity recommended by the World Health Organization are much longer during the first years of life compared to adulthood. However, many children do not meet these recommendations, which puts their current and future health at risk. Indeed, the earlier the lack of physical activity occurs, the greater the risk of the emergence of various pathologies in adulthood (Booth et al., 2012). Beyond the sedentary lifestyle, hypoactivity might be the consequence

of accidents or illnesses requiring prolonged bed rest. In addition, reduction in physical activity and low interaction with the environment is a common observation in children that display developmental coordination disorder (Paquet et al., 2019; Vaivre-Douret et al., 2016). Developmental coordination disorder, which concerns about 5–6 % of age-school children (WHO, 2023), is a neurodevelopmental disorder corresponding mainly to motor impairment ranging from gross to fine skill deficits that interfere with daily social and academic activities. Developmental coordination disorder generates an abnormal/atypical sensorimotor experience and low interactions with the environment that impact the entire sensorimotor pathway. Due to the existence of close relationships between muscle and central nervous system, sensorimotor restriction generates an atypical sensory input that affects the somato-sensory pathway. In return, motor command is altered, which affects muscle properties (Canu et al., 2019). Thus, brain and muscle communicate through a deleterious, self-sustaining cycle: abnormal movements would affect the immature nervous system, which would have

*Abbreviations:* BW, body weight; CTRL, control; MWW, muscle wet weight; SMR, sensorimotor restriction.

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consequences on the muscular system and would reinforce the production of abnormal movements (Canu et al., 2019; Coq et al., 2020).

In rodents, the central nervous system is very immature at birth and physical activity is essential for its maturation. The latter triggers processes facilitating neuroplasticity such as neurogenesis, synaptogenesis, and angiogenesis (Hotting and Roder, 2013). During typical development, the repertoire of general movements in limbs becomes more complex with age until fluid, precise and increasingly complex movements are acquired (Hadders-Algra, 2018). The increased variation and complexity of these general movements allows the development and refinement of the sensorimotor networks. The acquisition of an adequate sensorimotor function requires the development of muscle system to ensure the support of the body weight, an efficient motor command, the maturation of vestibular system for equilibrium and of somatosensory feedback as well (Jamon, 2006). All these steps take place in the first weeks of life and changes in the activity or environmental conditions at this period, for instance by immobilization or exposure to hyper- or hypogravity, has irreversible effects, supporting the existence of critical periods in the neuromuscular development (Jamon, 2006; Visco et al., 2024; Walton et al., 1992). In the same way, early movement deprivation has deleterious consequences. The sensorimotor restriction model (SMR) consists of immobilizing pup hind limbs from postnatal day 1 (P1) to P28 (Coq et al., 2008; Strata et al., 2004). Briefly, SMR induces a muscle atrophy characterized by a reduction in fiber surface, loss of strength, persistence of neonatal and fast myosin heavy chain isoform to the detriment of the slow one; hyperreflexia of the lumbar spinal cord; cortical disorganization with reduction of cortical representation maps of the hind limb in both sensory and motor cortex and cortical hyperexcitability; alteration in sensorimotor tasks such as rotarod or locomotion in juvenile or adult rats (Canu et al., 2022; Coq et al., 2020; Delcour et al., 2018a; Delcour et al., 2018b).

In the present paper, our objective was to determine whether SMR affects the early postnatal reflex ontogeny. To that purpose, we used a series of tests to assess neurodevelopmental reflexes and determine the age of achievement of developmental milestones. These tests represent reliable indicators of neurological and behavioral development and can be used to highlight the reflexes of the spinal cord as well as the maturation of the cortical networks (myelination, synaptogenesis, etc.) (Fox, 1965; Nguyen et al., 2017; Visco et al., 2024). Neurodevelopmental reflexes include eyelid reflex, fore limb/hind limb grasping, hind limb placing, righting, negative geotaxis and cliff avoidance. We also evaluated the maturation of postural control. Our results demonstrate that a SMR situation induces a delay in the acquisition of the main neurodevelopmental parameters.

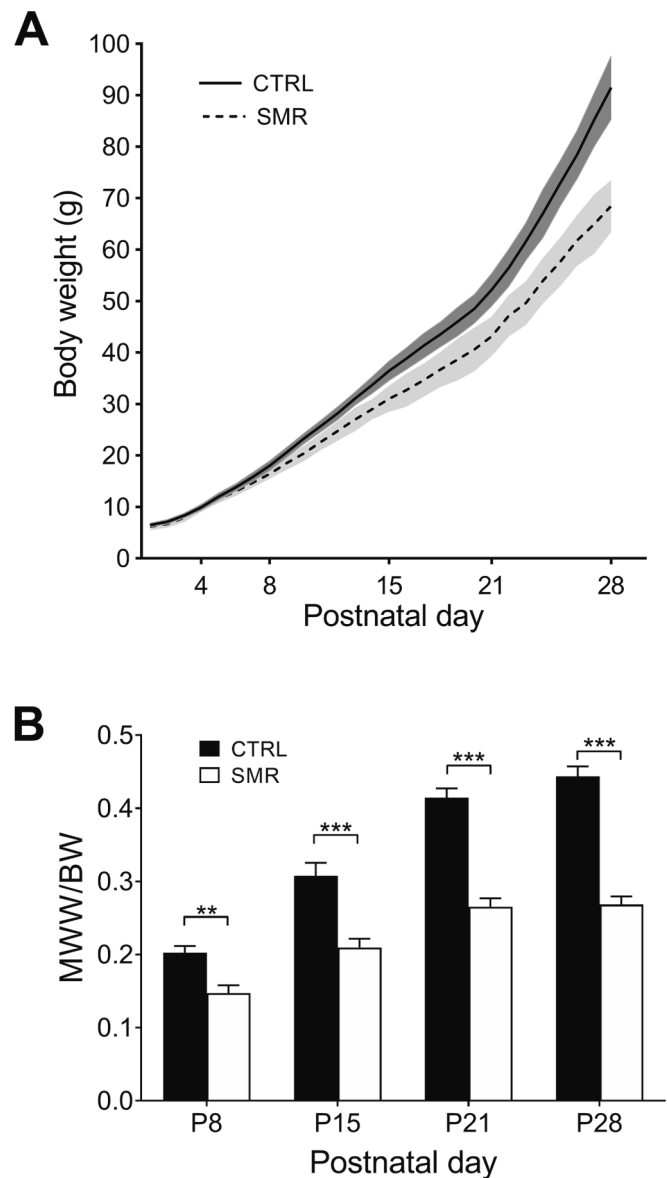
## 2. Results

Statistical analysis (Supplementary Table 1) revealed no effect of Sex. In addition, it highlights a Day effect for all tests except fore limb grasping (which was validated as soon as P2 for most rats). In consequence, both sexes will be pooled in the result description and we will focus on the Group effect.

### 2.1. General developmental observation

At birth, litters were randomly assigned to the CTRL or SMR groups. The body weight (BW) of pups was  $6.50 \pm 0.08$  g and  $6.45 \pm 0.13$  g in CTRL and SMR groups, respectively (*t*-test: ns) (Fig. 1A). At P28, BW increased to  $91.49 \pm 1.38$  g and  $68.50 \pm 1.58$  g for the CTRL and SMR groups, respectively. Compared to controls, SMR pups thus showed a growth retardation that reached  $-25\%$  at P28 (Group effect:  $\beta = -5.511$ ;  $p < 0.001$ ).

A previous study has provided evidence for a muscle atrophy at P28 (Canu et al., 2022). Thus, in a series of rats, we assessed whether soleus muscle was atrophied as soon as P8, and then at P15, P21 and P28. We observed a strong decrease in muscle wet weight (MWW) in SMR rats



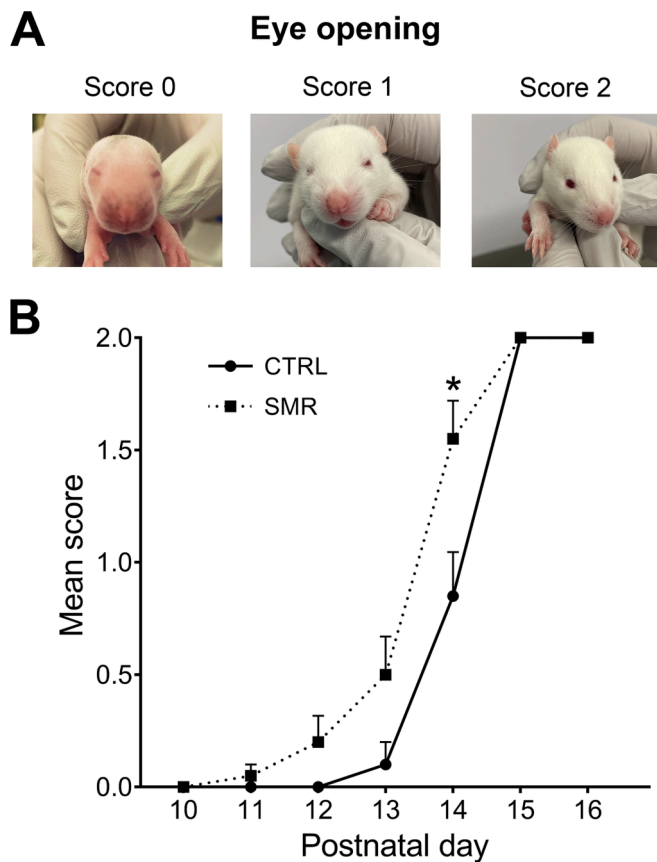
**Fig. 1.** Morphological parameters. A. Growth curve over time. Body weight was recorded daily from P1 to P28 for CTRL ( $n = 20$ ) and SMR ( $n = 20$ ) animals. Values are mean  $\pm$  SD. P6:  $p < 0.05$ ; P7:  $p < 0.01$ ; P8 to P28:  $p < 0.001$ , with respect to CTRL. B. Muscle wet weight / Body weight ratio for soleus muscle at P8, P15, P21 and P28. Muscles were sampled in 20 CTRL and 20 SMR at each age. Values are mean  $\pm$  SEM. \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$  with respect to CTRL.

(Group effect:  $e = -8.425$ ;  $p < 0.001$ ). When MWW was normalized to BW (Fig. 1B), a Group effect was still detected ( $e = -0.115$ ;  $p < 0.001$ ). The difference was significant at P8 ( $-27\%$ ,  $t = 4.035$ ,  $p < 0.001$ ) and become more pronounced over time (P15:  $-32\%$ ,  $t = 4.576$ ,  $p < 0.001$ ; P21:  $-36\%$ ,  $t = 8.779$ ,  $p < 0.001$ ; P28:  $-39\%$ ,  $t = 9.919$ ,  $p < 0.001$ ), indicating that SMR rats displayed a severe atrophy of soleus muscle, that cannot be attributed to the lower BW.

Eye opening began at P13 for CTRL whereas it began as soon as P11 for SMR pups (Fig. 2, Table 1). The difference tended to be significant (Group effect:  $e = +0.920$ ,  $p = 0.063$ ). However, all CTRL and SMR rats had both eyes opened at P15.

### 2.2. Effect of SMR on postural development

We first evaluated the postural development of pups, i.e. whether



**Fig. 2.** Eye opening. A. Photograph showing eye opening evolution on CTRL pups. Score 0 corresponds to no eyelid opened (P10), 1 to one eyelid opened (P13) and 2 to both eyelids opened (P15). B. Mean score from P10 to P16 obtained in 20 CTRL and 20 SMR animals. Values are mean  $\pm$  SEM. \*:  $p < 0.05$  with respect to CTRL.

**Table 1**

Postnatal day of maturation of postural development and neurodevelopmental reflexes. The day of validation is presented as median (min–max). Statistical comparison was carried out using Mann-Whitney test. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

Measure	CTRL	SMR
Eye opening	15 (13–15)	14 (11–15) *
Posture	14 (11–15)	18 (15–20) ***
<b>Neurodevelopmental reflexes</b>		
Fore limb grasping	2 (2–2)	2 (2–4)
Hind limb grasping	4 (2–8)	5 (2–10) **
Hind limb placing	5 (2–9)	3.5 (2–10) ***
Surface righting reflex	2 (2–6)	2 (2–8)
Cliff avoidance	3 (2–6)	6 (2–10) ***
Negative geotaxis	12 (5–13)	17 (11–20) ***
Eyelid reflex	2 (2–3)	2 (2–4)

they can extend their limbs to lift the abdomen from the surface and exhibit locomotor movements. We observed two main phases in postural support acquisition (Fig. 3). First, CTRL rats evolved from a score of 0 (i. e. no movement) to 1 (crawling without pelvis elevation) from P1 to P3. At P4, the crawling step was acquired for 100 % of CTRL animals. The second phase extended from P11 to P14 where pups progressively reached the maximum score of 2, corresponding to a mature posture with limb support. In SMR rats, acquisition of postural control was delayed ( $\beta = -1.309$ ,  $p < 0.001$ ). The first phase was prolonged until P8 whereas the second phase began at P15 and lasted until P20. In

particular, we observed that hind limbs were frequently extended, lying passively with dorsal paw in contact with the floor, i.e. without plantar placement. Mature bilateral plantar placement occurred later in SMR rats. Thus, the mean score reached a maximum value of  $2.0 \pm 0.0$  for CTRL rats at P15 whereas it was only  $1.2 \pm 0.1$  ( $p < 0.001$ ) for SMR rats at this age (Supplementary material, Fig. S1-A). In summary, the acquisition of crawling (first phase) and of limb support (second phase) were delayed by 4 and 5 days respectively for SMR.

### 2.3. Effect of SMR on neurodevelopmental reflexes

#### 2.3.1. Grasping

Most rats exhibited the grasping reflex with one or two fore limbs very early since all CTRL rats obtained the maximum score at P2 (data not shown) while SMR rats completed the test at P4 (Supplementary material, Fig. S1-B). The difference was not significant.

Achievement of hind limb grasping reflex appeared later (Fig. 4). At P2, the reflex was acquired for both hind limbs in 15 % of CTRL rats vs. only 5 % of SMR ones and the mean score (Supplementary material, Fig. S1-C) was significantly lower by 41 % for SMR pups. At P4, 74 % of CTRL animals obtained a score of 2 (mean score:  $1.75 \pm 0.07$ ) against 49 % for SMR (mean score:  $1.35 \pm 0.12$ ,  $p < 0.05$ ). SMR group totally validating this test with a 2-day delay (P10 vs. P8). However, statistical analysis revealed no difference between groups (Group effect for mean score:  $\beta = -0.490$ ;  $p = 0.537$ ).

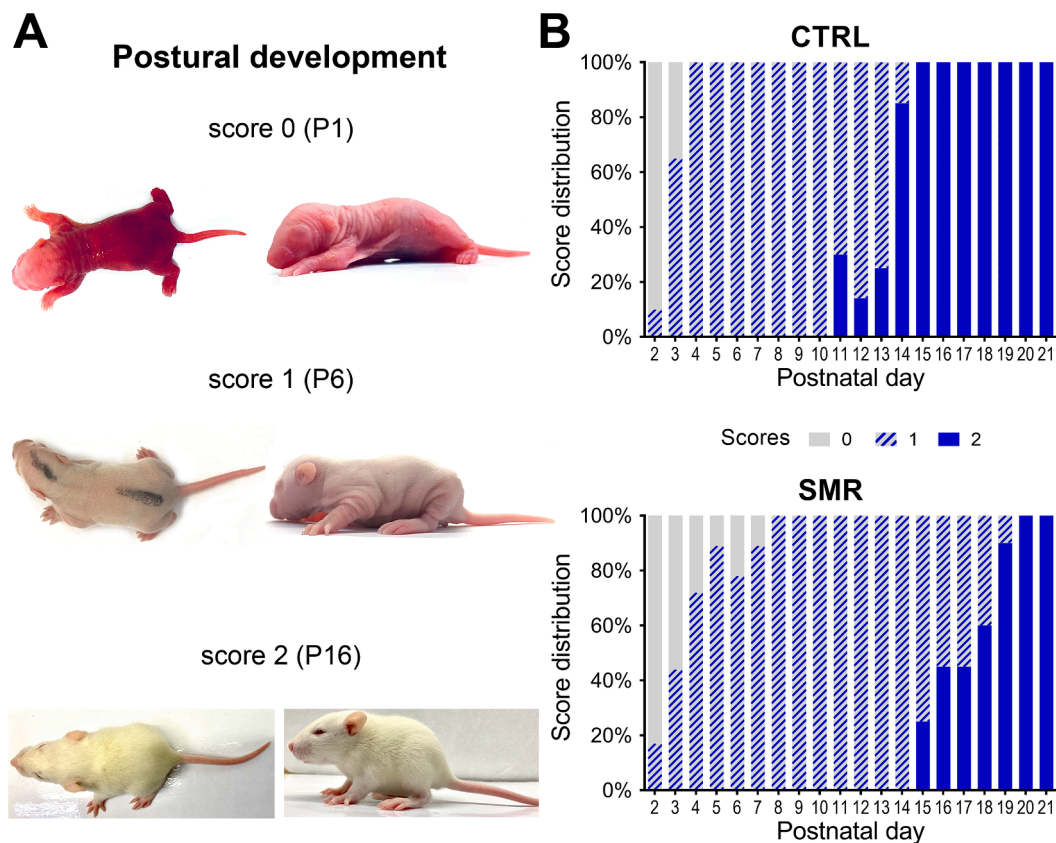
#### 2.3.2. Hind limb placing

The back of the foot was touched with the edge of a flat surface while the animal was suspended by the torso (Fig. 5A). A correct placing reflex corresponds to a paw withdrawal followed by contact on the surface after the dorsal face has been put in contact with the edge of that surface. A response was detected at P2 for a few CTRL rats. It consisted mainly of a paw withdrawal, without paw placement. However, 5 % of CTRL and 2 % of SMR rats exhibited a correct placing reaction as soon as P2 (Fig. 5B). The mean score at P2 was then  $0.27 \pm 0.09$  and  $0.19 \pm 0.1$  in CTRL and SMR groups, respectively (n.s.) (Supplementary material, Fig. S1-D). In CTRL rats, the evolution of scores over time followed a sigmoid curve to reach a maximum value at P9. Multiple linear regression model showed no Group effect ( $\beta = 0.017$ ,  $p = 0.970$ ). However, in SMR rats, the score increased very strongly from P2 to P3: at P3, SMR animals have a significantly higher mean score than CTRL (+214 %,  $p < 0.001$ ). At P4, the mean score of SMR group was still significantly higher (+75 %,  $p < 0.05$ ), with 60 % of SMR pups validating the test against only 24 % of CTRL animals. From P5, there was no longer any significant difference between the 2 groups. Nevertheless, 100 % of SMR pups passed the test at P12 while this test was completely validated at P9 for CTRL pups.

#### 2.3.3. Surface righting reflex

The surface righting reflex evaluates the capacity of the animal to roll over and to recover a prone position when placed in a supine position in less than 15 s (Fig. 6). At birth, pups first exhibited an ineffective corkscrew tactic (Harding et al., 2017), in which the head and shoulder rotate in one direction while pelvic girdle rotate in the opposite direction. Then, a mature axial tactic appeared, with shoulder and pelvic girdles rotating in the same direction. At P2, 78 % of CTRL rats validated the test with a mean time of  $8 \pm 1$  s in CTRL group. Rats in CTRL group showed a progressive and steady increase in performance between P2 and P6, when considering for the percentage of animals validating the test (Fig. 6B) as well as the mean score (Supplementary material, Fig. S1-E). At P6, 100 % of rats were able to recover a prone position with a mean duration of  $3 \pm 1$  s. In SMR rats, no difference was detected in score (Group effect:  $\beta = -0.192$ ,  $p = 0.795$ ) with respect to CTRL group, despite the fact that some animals did not validate the test before P9, i.e. with a 3-day delay.

Rats were tested until they obtained the maximum score for two



**Fig. 3.** Effect of SMR on postural development. A. Photograph showing pup characteristic posture (CTRL pups). Score 0 corresponds to an absence of movements, score 1 to abdomen dragging with paws perpendicular to the body, and score 2 to mature posture (elevated abdomen, limbs parallel to the body). B. Stacked bar chart showing the distribution (%) of the scores from P2 to P21. Data obtained in 20 CTRL and 20 SMR animals.

consecutive days. When the time to achieve the test for these two last essays was compared, we observed that the righting duration was higher in SMR rats than in CTRL ones (+50 %,  $p < 0.01$ ) (Fig. 6C). Video recording reveals that, although the maximum score was reached and then the test was achieved, SMR had difficulties in coordinating shoulder and pelvic girdles and in acquisition of the axial tactic, and they persisted longer in the corkscrew strategy (Supplementary material, Video S1).

#### 2.3.4. Cliff avoidance

The cliff avoidance test evaluates a reflex to avoid a dangerous situation, in this case to avoid falling into the void (Fig. 7A). At P2, 63 % of CTRL animals and 44 % of SMR ones detected the cliff and tried to move away from it (score 1, Fig. 7B). The strategy consisted to rotate the whole body by pushing on fore and hind limb on one side (most often the right side) to move the body toward the opposite side, and then lifting the other limbs to place them further away (supplementary material, Video S2). 15 % of CTRL animals obtain the maximum score (corresponding to rotating followed by moving away from the edge) as soon as P2 against 5 % in SMR pups. The reflex was acquired for all rats at P6 for CTRL and P10 for SMR rats. The delay can be explained by the fact that for some SMR rats, hind limbs were lying passively and pups had no body support, and thus cannot exert a push. Other rats seem to present a deficit in the independent control of hind limbs. In that case, they exerted a bilateral push, moving the body forward instead of rotating. The mean score was lower in SMR rats (Group effect:  $\beta = -1.968$ ,  $p < 0.001$ ) (Supplementary material, Fig. S1-F). In CTRL rats, for the two last essays, the test was achieved in  $9.6 \pm 0.7$  s and the latency was increased in SMR rats (+52 %,  $p < 0.001$ ).

#### 2.3.5. Negative geotaxis

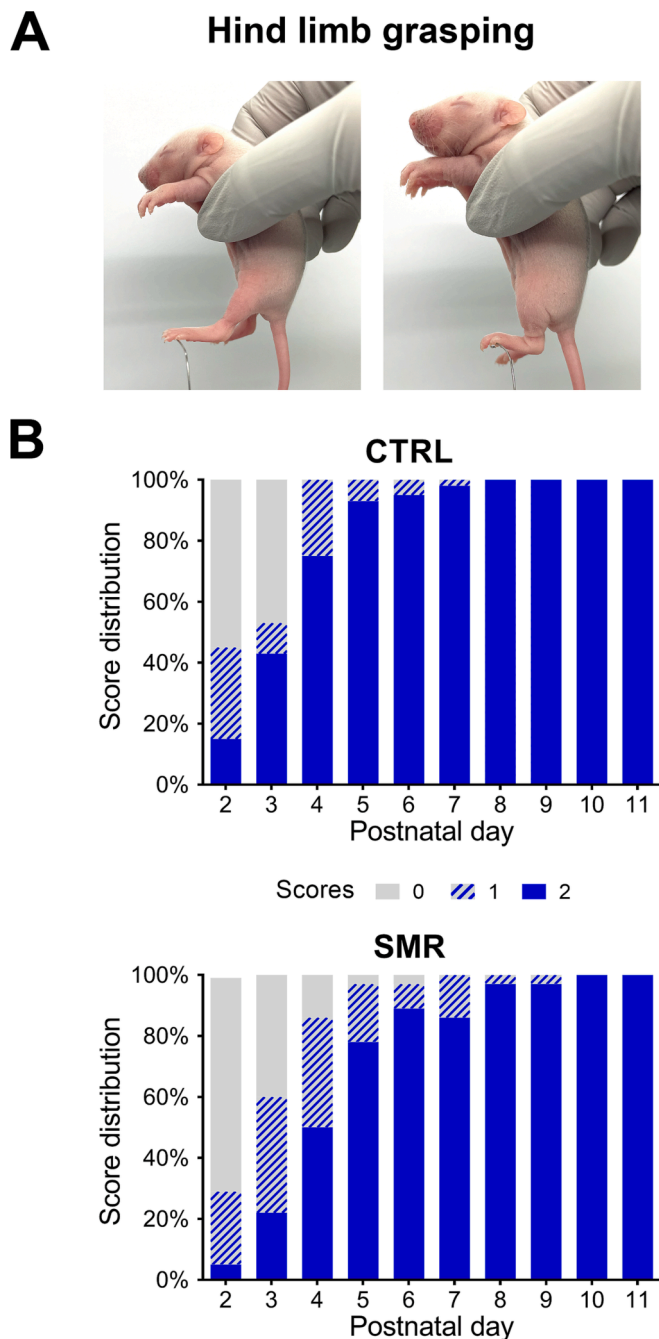
Negative geotaxis is a test classically used to evaluate vestibular system and to measure sensorimotor ability in pups. Animals were left 30 s on an inclined platform and their capacity to rotate and climb was observed (Fig. 8A).

Some animals began to move and to rotate at P2, suggesting that they correctly detected the slope (25°) (P2, score 1: 40 % in CTRL, 20 % in SMR,  $\chi^2$ :  $p < 0.001$ ) (Fig. 8B). This performance was rapidly improved in CTRL rats, since more than 80 % reached a score of 1 as soon as P4 whereas this percentage was not reached before P9 in SMR ones. Video recording analysis showed that most SMR rats suffered from a lack of stabilization of their hindquarter, generating a lateral instability (Supplementary material, Video S3). This problem was due to the absence of plantar support on hind limbs as well as to a poor coordination between hind limbs. As a result, the pups fell and rolled down the inclined platform when they tried to turn around to get the head up.

In CTRL rats, there was a step at P5, where 30 % of pups suddenly reached the score of 2, corresponding to platform climbing. Such a step was also observed in SMR rats, but later on, at P12. Finally, all CTRL rats succeeded in the test at P13 while it was not until P20 for SMR rats, thus with a 7-day delay.

The difference between CTRL and SMR score was significant from P3 until P17 (Group effect:  $\beta = -2.520$ ,  $p < 0.001$ ) with a mean score for SMR rats corresponding to ~60 % of CTRL one (Supplementary material, Fig. S1-G). The mean time to achieve the test was  $16.1 \pm 0.8$  s in CTRL rats. It was greatly increased in SMR ones (+37 %,  $p < 0.001$ ).

In conclusion, there is a delay in acquisition of main reflexes (hind limb grasping: +2 days, righting and hind limb placing: +3 days, Cliff avoidance: +4 days, negative geotaxis: +7 days) and an alteration in motor performance (time to perform the test: +37 to +52 % increase).



**Fig. 4.** Effect of SMR on grasping reflex. A. Photograph showing hind limb reflex testing on CTRL pup at P10. A score of 0, 1 and 2 corresponds to a grasping reflex observed in 0, 1 or 2 hind limbs respectively. B. Stacked bar chart showing the distribution (%) of the scores from P2 to P11 for hind limb grasping reflex. Data obtained in 40 CTRL and 40 SMR animals.

### 2.3.6. Eyelid reflex

Eyelid reflex appeared within the first days. At P2, the reflex was already present bilaterally in ~80 % of rats (mean score:  $1.7 \pm 0.1$  for CTRL;  $1.8 \pm 0.1$  for SMR, [Supplementary material, Fig. S1-H](#)). Animals reached the maximum score (i.e. 2) as soon as P3 for CTRL and P4 for SMR rats. No difference was detected between groups ( $\beta = 0.082$ ,  $p = 0.884$ ).

## 3. Discussion

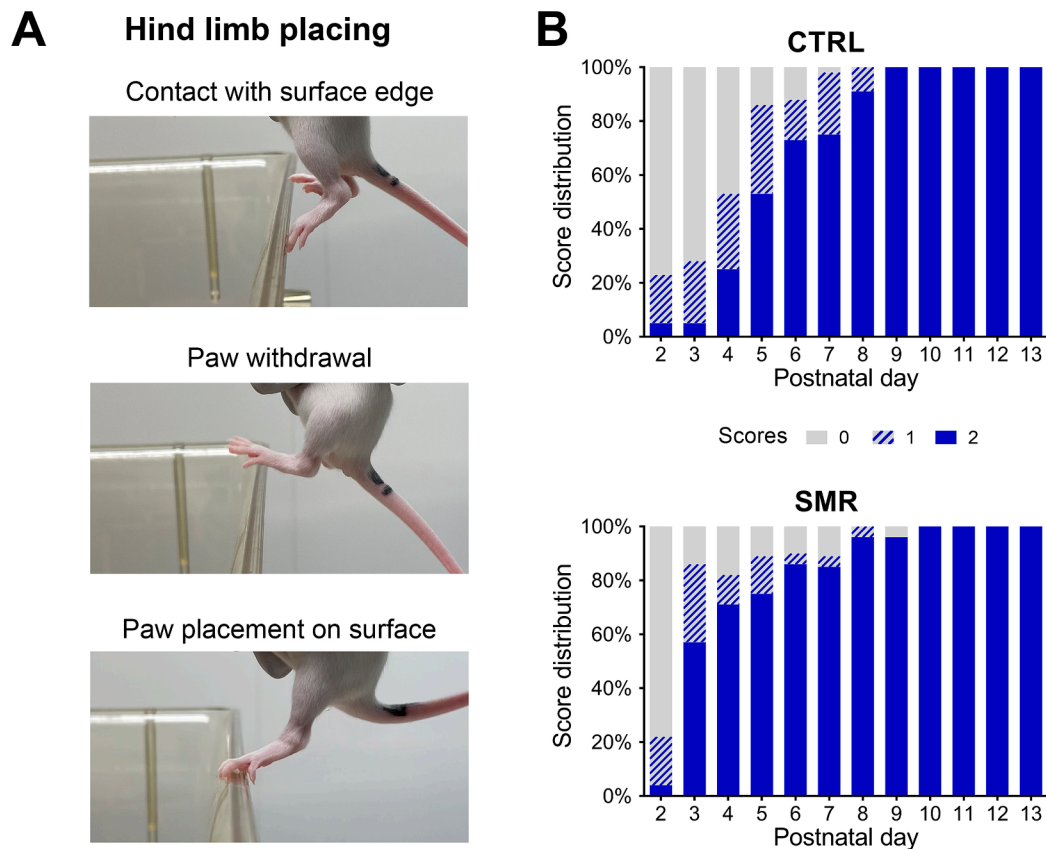
Previous studies showing deleterious consequences of SMR on the

neuromuscular system were conducted after the 28-day restriction period ([Canu et al., 2022](#); [Delcour et al., 2018a](#); [Delcour et al., 2018b](#)). Our objective was to assess the pup's development from P1 to P28. We explored the development of the nervous system through the determination of the age for achievement of the main neurodevelopmental reflexes. We demonstrated that SMR affects the early postnatal reflex ontogeny. In particular, it induces a delay in the motor development, illustrated by a several days delay in the acquisition of a mature posture and in the acquisition of several reflexes: hind limb grasping (+2 days), righting (+3 days), hind limb placing (+3 days), cliff avoidance (+4 days), negative geotaxis (+7 days). The later the neurodevelopmental tests were validated after birth, the greater the delay for the SMR group ([Fig. 9](#)). In addition, the time to perform the test was increased (+37 % for negative geotaxis, +52 % for cliff avoidance), suggesting an alteration of performance. We also observed that SMR induced a body growth restriction with a severe atrophy of soleus muscle, and an earlier eye opening.

### 3.1. Primitive reflexes are delayed

This study first indicates that SMR can have consequences in the early stages of development, i.e. in the very first days after birth, at a period where motor activity of pups is restricted to episodic spontaneous twitch-like movements ([Robinson et al., 2000](#)). This result is in accordance with [Visco et al. \(2024\)](#), who also reported a delayed motor development in a rat model of cerebral palsy, combining SMR and perinatal anoxia. Secondly, it also indicates that the whole P1-P15 period is sensitive for acquisition of developmental milestones. Indeed, all reflexes whose maturation normally occurs within this developmental period are affected by SMR. Moreover, sensorimotor reflexes devoid of postural support and/or that do not require high levels of force development (fore- and hind limb grasping reflex, placing, eyelid) are less affected by SMR than developmental tests requiring whole body movement (righting reflex, cliff aversion and negative geotaxis test) with limb coordination.

The grasping (fore- and hind limbs) and placing reflexes are primitive reflexes that appears very early and then disappear during postnatal development ([Fox, 1965](#); [Laliberte et al., 2021](#)); they are frequently used in children to detect pathologies such as cerebral palsy ([Zafeiriou, 2004](#)). Primitive reflexes depend on spinal circuits that are refined during development by descending pathways, sensory feedback and maturation of intrinsic properties of spinal neurons ([Clarac et al., 2004](#)). In humans, grasping can be observed in utero, in particular by grasping the umbilical cord ([Jakobovits, 2009](#)). We observed in the present study that grasping appeared first in fore limbs and then in hind limbs. Such a rostrocaudal gradient in maturation of spinal cord is well known. It reflects the maturation of the corticospinal tract with cervical spinal cord reaching functional maturity earlier than lumbar one ([Vinay et al., 2000](#); [Vinay et al., 2002](#)). The growth of corticospinal axons is also in close relationship with the appearance of the placing reflex ([Donatelle, 1977](#)). The importance of primary motor cortex (M1) activity in shaping the early postnatal refinement of corticospinal terminations is well established. Indeed, in cat, inactivation of the motor cortex by GABA agonists during the infantile period results in aberrant pattern of cortical projections within the spinal cord ([Martin et al., 2007](#) for review). In SMR rats, the delay in reflex appearance might reflect a change in the refinement of spinal circuitry due to a change in M1 activity ([Delcour et al., 2018b](#)). In the same way, the organization of spinal network is dependent on peripheral activity since muscle blockade by Botulinum toxin A in newborn cats affects corticospinal projections ([Martin et al., 2007](#)). Thus, the atypical sensorimotor experience (absence of postural support and of stepping movements of hind limbs) in SMR rats might induce changes in proprioceptive feedback and in consequence affect the organization of corticospinal endings.



**Fig. 5.** Effect of SMR on hind limb placing. A. Photograph showing hind limb placing reflex on CTRL pup at P14. B. Stacked bar chart showing the distribution (%) of the scores from P2 to P13 for hind limb placing. Data were obtained in 35 CTRL and 30 SMR animals. Score 0, 1 and 2 correspond to a placing reflex observed in 0, 1 or 2 hind limb respectively.

### 3.2. Postural control is dependent on muscle maturation

In CTRL rats, we observed steps in postural support acquisition: the first milestone was reached at P4 where all pups exhibited walking-like movement (crawling) with limbs in exorotation but were unable to support their body weight, and the last step at P15 where all pups exhibited a mature posture with elevation of the pelvis, adduction of hind limbs and fluent locomotion. This observation is in accordance with Geisler et al. (1993) who described three periods in postural development and observed the acquisition of mature posture at P15. For SMR animals, a delay was observed in the acquisition of the two steps, leading to the appearance of a mature posture at a later age.

Postural development depends on several parameters such as the capacity of postural muscle to generate enough force to counteract gravitational force. We have shown a dramatic atrophy of the soleus postural muscle as soon as P8. Thus, the retardation in postural support in SMR rats might be the consequence of muscle weakness. However, in a previous study one (Canu et al., 2022), we have shown that despite a strong atrophy of the postural soleus muscle at P28, the specific force (tension / muscle wet weight) was unchanged. In addition, in vivo, the force developed by hind limb on the grip test was strongly reduced in SMR rats but when normalized to body weight, value was like CTRL. Thus, we formulate the hypothesis that the delay in mature posture acquisition also depends on the maturation of spinal network.

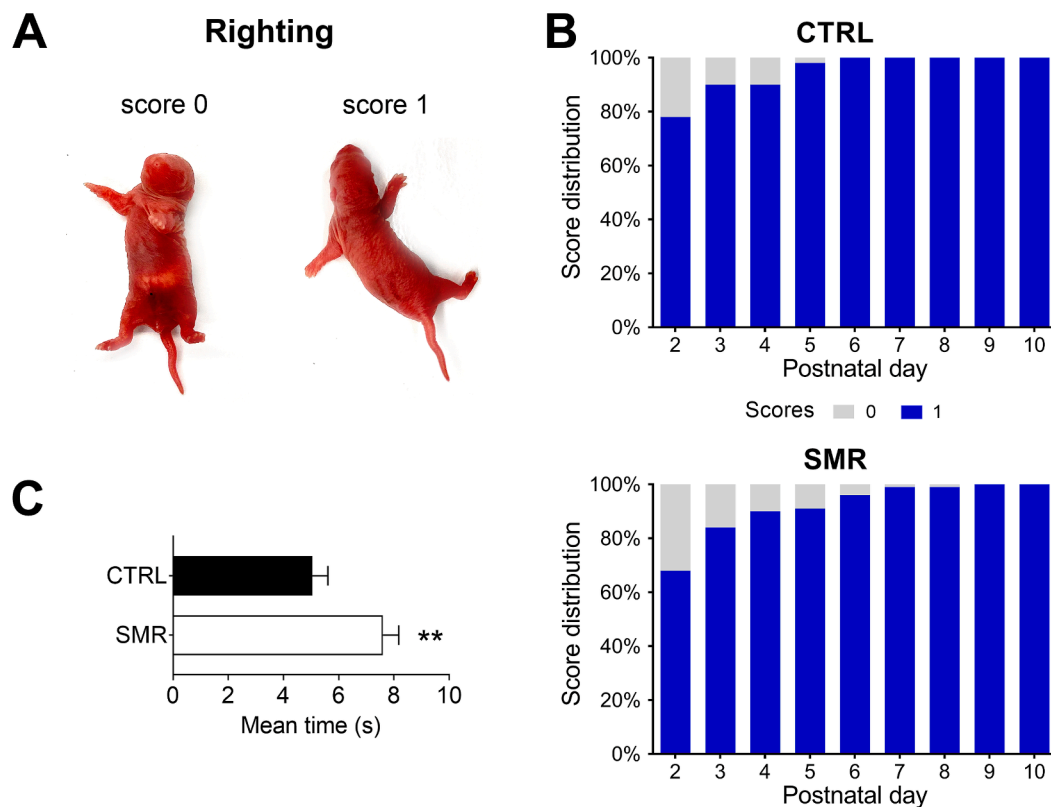
### 3.3. Development of complex motor behavior

Postural control is considered the limiting factor for complex motor behavior (Lelard et al., 2006; Westerga and Gramsbergen, 1993). Righting, cliff avoidance and negative geotaxis are tests that necessitate multisensory integration, involving in particular the vestibular system to

perceive head position (Altman and Sudarshan, 1975), and require muscle force for moving as well as a good motor coordination. The delay in the appearance of these reflexes and mature posture in SMR pups could suppose a motor deficit and/or a defect in the integration of sensory information. However, as already mentioned, force loss was moderate in SMR rats at P28 (Canu et al., 2022). Thus, we assume that abnormal proprioceptive input is more important than force development in affecting these specific motor behaviors. Video recording analysis has demonstrated that SMR pups exhibited erratic movements with poor interlimb coordination, which affects their capacity to turn back in the cliff avoidance and negative geotaxis tests.

Given the importance of spinal input in the experience-dependent maturation of spinal circuits, the role of proprioceptive afferent input should be questioned. Proprioceptive afferent fibers from muscle spindles report the variations in muscle length to the spinal cord through monosynaptic reflex. The spinal connections of Ia sensory input onto motoneurons are set up during the prenatal period; they exist at birth and they are strengthened postnatally (Imai and Yoshida, 2018). Thus, the monosynaptic reflex is functional at birth, and there is an activity-dependent refinement of the spinal circuitry in the early postnatal period. During SMR, the hind limbs are immobilized in extended position, and consequently, muscle spindles from extensor muscles (mostly postural) are not solicited. Mendelsohn et al. (2015) have shown in mice that genetically abolishing neurotransmission from proprioceptive sensory neurons onto motoneurons strongly affects the number and density of connections on synergist motoneurons as soon as P7.

Postnatal change in motoneuronal firing properties and excitability is also a key-factor for appearance of mature posture, in particular, differential maturation of motoneurons innervating the flexor and extensor muscles of the hind limbs (Vinay et al., 2002). To our knowledge, there is no evidence that this maturation is activity-dependent.



**Fig. 6.** Effect of SMR on righting reflex. A. Photograph showing righting reflex on a CTRL pup at P2. Score 0 corresponds to a pup that stayed in supine position. Score 1 corresponds to a pup that recover a prone position. B. Stacked bar chart showing the distribution (%) of the scores from P2 to P10 for body righting. Data were obtained in 40 CTRL and 70 SMR animals. C. Histogram showing the mean time for achieving the test for the two last essays (the reflex was considered as acquired when a rat succeeded for two consecutive days and individual was not tested anymore). \*\*:  $p < 0.01$ ,  $t$ -test.

However, sensory input from large diameter fibers, i.e. proprioceptive sensory information from muscle spindles, plays a substantial role in the morphological and physiological maturation of the motoneurons (Tahayori and Kocaja, 2012). Therefore, SMR likely affects the maturation of sensorimotor connectivity and hence disrupts the development of sensorimotor integrative reflexes.

In addition, using genetic perturbation experiments, Basaldella et al. (2015) have demonstrated that interactions between the proprioceptive and vestibular system shape the organization of vestibular projections onto the spinal cord motoneurons. The vestibular system is effective from P1 and requires about 2 weeks to mature. The slow extensor soleus is the main target of vestibulospinal projections in wild-type mice. However, in *Egr3* mutant mice, that exhibit early postnatal degeneration of muscle spindles, leading to non-functional muscle spindle afferents, more vestibulospinal fibers connect to motoneurons innervating fast gastrocnemius muscle. Thus, proprioceptive input shapes vestibular projection to specific motoneuron subtypes. It is also established that hind limb proprioceptive input and vestibular information are integrated within vestibular nuclei in adult cat (McCall et al., 2021), but whether this integration is affected by an early sensorimotor restriction remains to be determined. However, functional interaction with proprioceptive circuitry is necessary to ensure smooth motor behavior, and these data demonstrate that an abnormal sensorimotor input induces an abnormal organization of spinal cord circuitry. It may explain why realization of complex motor behavior for offspring, such as cliff avoidance, righting or negative geotaxis, are delayed, and even when the reflex is acquired, it takes longer to complete in SMR pups.

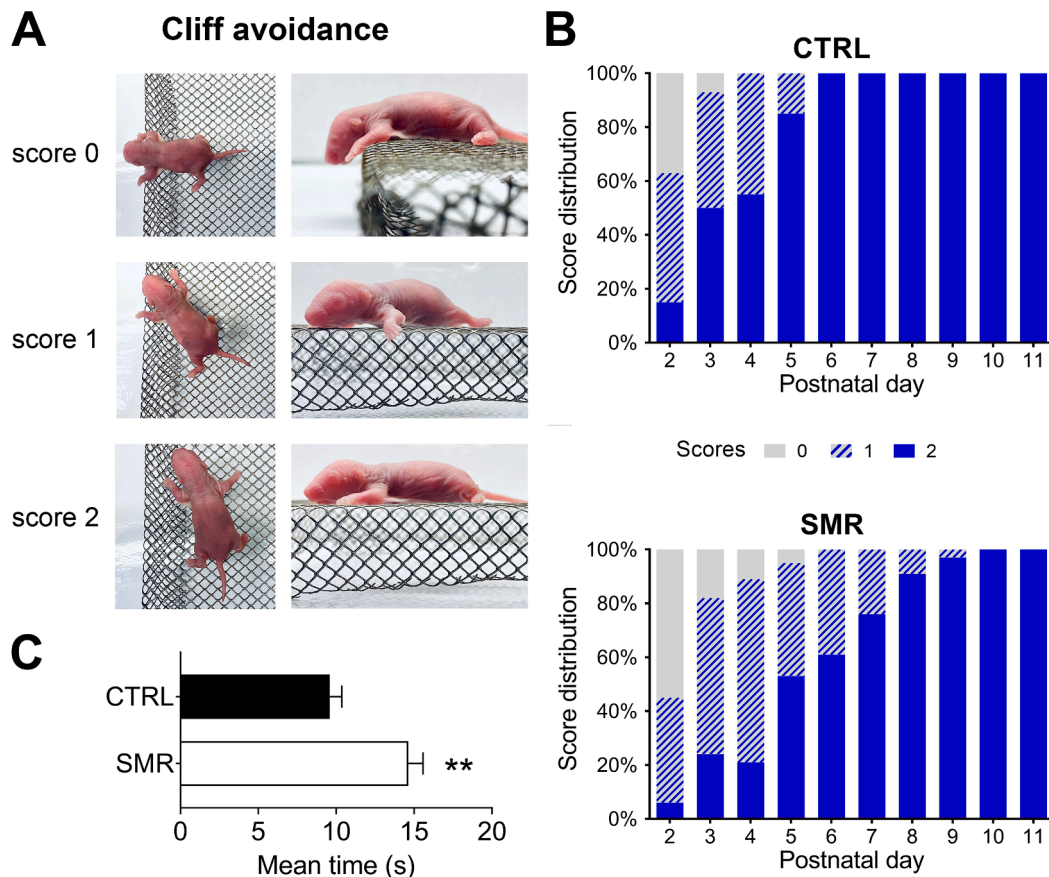
### 3.4. Dam – pups interactions

The results highlighted in this study and in particular the delays in

the onset of reflexes were attributed to SMR. However, the role of maternal care can be considered. Indeed, at an early age, maternal care represents the main source of sensory and social stimulation. In addition, maternal separation early in life and handling are considered stressful (Bolton et al., 2017). Thus, a change in maternal care and/or pup stress could influence the onset of reflexes. Yang et al. (2021) have shown that when the pups were separated from the mother from P1 to P14, 6 h/day during the light phase, the percentage of animals validating the cliff avoidance test at P14 was lower in the group deprived of maternal care but on the other hand, no delay in the righting time nor in the reversal time for the negative geotaxis was observed. In the present study, the duration of the separation for immobilizing hind limbs did not exceed 15 min per day, and the CTRL rats were also separated from the mother and handled by the experimenter. Furthermore, neonatal handling in rats enhances licking/grooming behavior when pups are returned to the nest (González-Mariscal and Melo, 2017). Finally, we did not observe any change in the weight of adrenals at P15 and P28 (unpublished results), suggesting that there was no elevation in stress level due to SMR procedure.

The body weight was much lower in SMR rats than in CTRL ones. Other studies also found a lower weight of SMR pups (Canu et al., 2022; Delcour et al., 2018a; Delcour et al., 2018b). This result could be explained by difficulty in accessing the nipples for SMR pups that could lead to a decrease in food intake, due to the cast clutter or to motor deficits. Indeed, during the first 2 weeks of life, pups feed exclusively through breastfeeding. Furthermore, attachment to nipples has been shown to require different postural adjustments (Eilam and Smotherman, 1998) and we demonstrated here that SMR delays postural maturation. However, during the dark period, dams are more active and in consequence are less involved in maternal care (nursing, licking, grooming) than during the light period (Champagne et al., 2003; Ivy





**Fig. 7.** Effect of SMR on cliff avoidance. **A.** Photograph showing cliff avoidance test on CTRL pup at P3. Score 0 corresponds to a pup that did not move or fell, score 1 was assigned when the pup tried to move away from the cliff and test was validated (score 2) when the pup completely turned away from the cliff. **B.** Stacked bar chart showing the distribution (%) of the scores from P2 to P11 for cliff avoidance. Data were obtained in 40 CTRL and 35 SMR animals. **C.** Histogram showing the mean time for achieving the test for the two last essays (the reflex was considered as acquired when a rat succeeded for two consecutive days and individual was not tested anymore). \*\*:  $p < 0.01$ ,  $t$ -test.

et al., 2008). Therefore, immobilizing pups during the dark period has less impact on their food intake. In addition, Champagne et al. (2003) reported no difference in growth of the offspring at P22 according to the maternal behavior (high or low licking/grooming–arched-back nursing).

#### 4. Conclusion

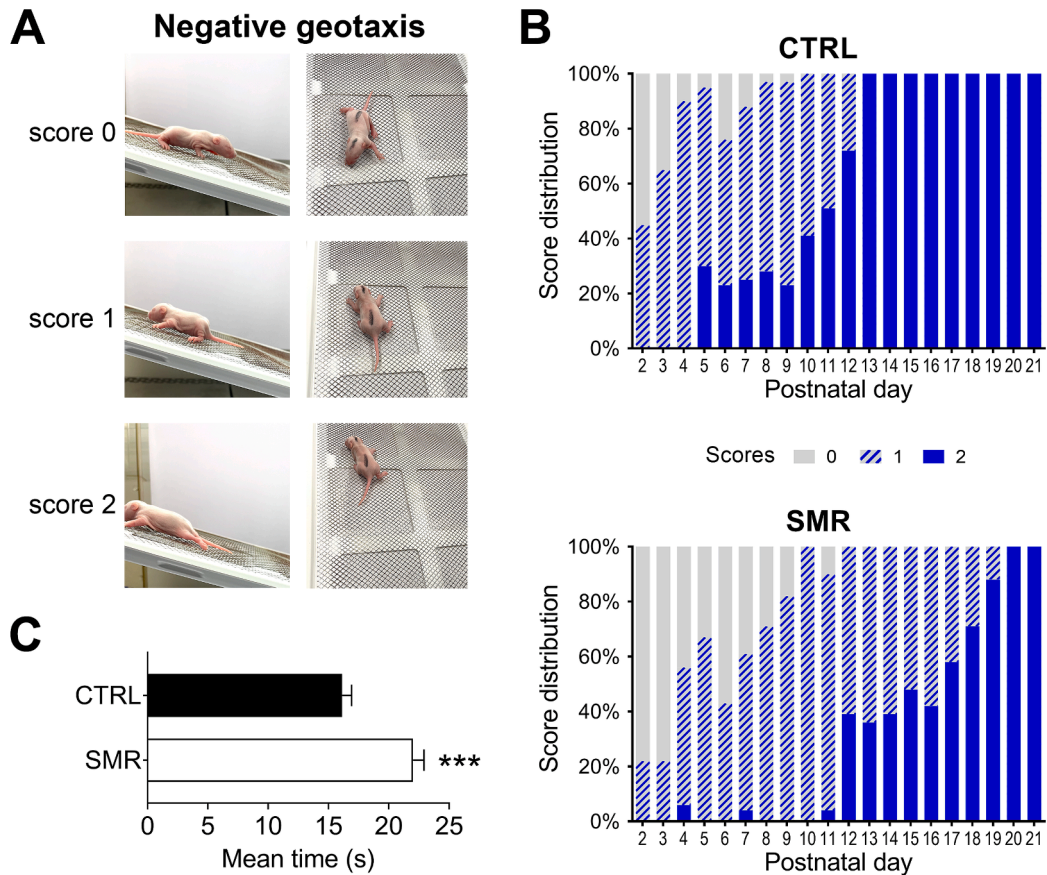
These data suggest that the first postnatal weeks are important for sensory inputs integration and the maturation of reflexes. It is estimated that the brain of a rat pup from P1 to P10 has reached a cerebral maturation corresponding to that of the human fetus during the last trimester of gestation (Workman et al., 2013). These weeks correspond to a critical period during which significant cortical maturation occurs, where the spinal circuits are established and where the mono-innervation of the myofibers sets in (Bennett and Pettigrew, 1974). In SMR pups, sensorimotor disruption alters the structuring and maturation of the nervous system and leads to abnormal cortical connections (Delcours et al., 2018b; Stigger et al., 2011). This results in delays in neurodevelopmental reflexes, as observed in the present study. A delay in acquisition and disappearance of these primitive reflexes, associated with various motor disturbances, has been observed in several neuro-behavioral disorders including developmental coordination disorder, attention deficit hyperactivity disorders or autistic spectrum disorders (Melillo et al., 2022), suggesting that the SMR model is relevant to study motor development in these children. Furthermore, SMR pups exhibited a delay in acquisition of developmental milestones even though immobilization was discontinuous and lasted only 16 h per day. During the 8-

hour activity period, rats exhibited atypical movements, with “toe walking” and increased variations in gait kinematic parameters (Delcours et al., 2018a), that persist over time. Thus, our observations highlight the importance of both regular physical activity and proficient movement execution throughout childhood. Given the importance of physical activity for motor skill development and the strong relationship between motor competence and physical activity, further studies should be conducted to decipher the link between muscle and central nervous system. In particular, we have observed that FNDC5/irisin expression level in the soleus muscle exhibited a peak at P15 in control as in SMR rats (unpublished data). FNDC5/irisin is a protein expressed in several organs including the skeletal muscle in response to exercise, supposed to be a key actor of muscle-brain communication. In the hippocampus, irisin induces the expression of the Brain-Derived Neurotrophic Factor (BDNF) (Wrann et al., 2013), a pivotal neurotrophin for brain development (Kowiański et al., 2018). A better understanding of mechanisms involved in the delay in neurodevelopmental reflexes could be useful to develop countermeasures to allow a proper maturation of primitive reflexes in children with an abnormal/atypical sensorimotor experience, which is the prerequisite for further acquisition of efficient motor skill performance.

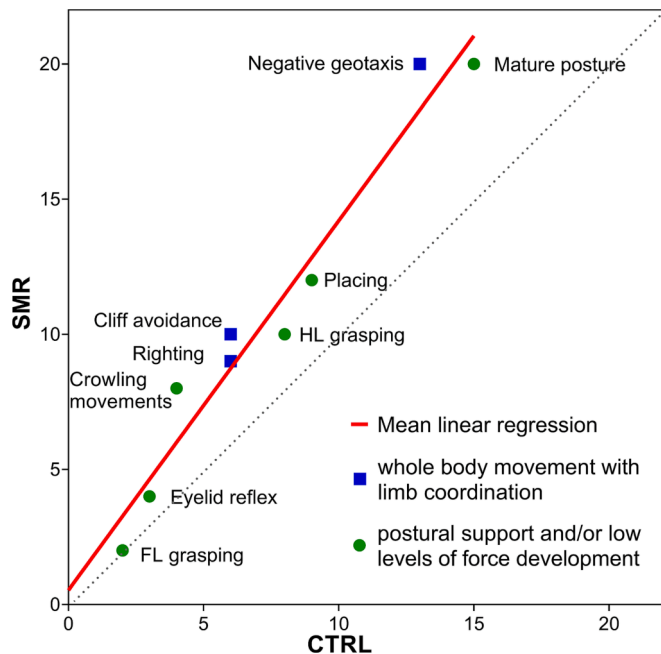
#### 5. Experimental procedure

##### 5.1. Animals

Experiments were performed on CD-Sprague-Dawley rats. Animals were housed in standard cages under adequate humidity, temperature,



**Fig. 8.** Effect of SMR on negative geotaxis. A. Photograph showing negative geotaxis test (slope = 25°) on a CTRL pup at P10. Score 0 corresponds to a pup that did not move, score 1 to a pup that turned and the test was achieved (score 2) when the pup turned and climbed. B. Stacked bar chart showing the distribution (%) of the scores from P2 to P21 for negative geotaxis. Data were obtained in 40 CTRL and 30 SMR animals. C. Histogram showing the mean time for achieving the test for the two last assays (the reflex was considered as acquired when a rat succeeded for two consecutive days and individual was not tested anymore). \*\*\*:  $p < 0.001$ ,  $t$ -test.



**Fig. 9.** Delay of primary reflexes in SMR vs CTRL rats. The figure shows that reflexes were achieved later in SMR rats, and the higher the age of acquisition, the greater the delay. The slope is  $1.37 \pm 0.12$  (different from 1,  $p < 0.01$ ).

and light conditions (22 °C, 51 % humidity) with a 12:12-h light-dark cycle. They had free access to water and food. Female and male were purchased from Charles River Laboratories (L'Arbresle, France). Following a 7-day acclimatization, a male was placed in a cage with 2 nulliparous females aged between 90 and 120 days each evening. After mating with a male rat (verified by a vaginal lavage containing sperm), pregnant female rats were housed into individual cages. Parturition day was P1. Pups were weighted when birth was detected and after their first feed. Females were examined for litters regularly from 8 AM to 6 PM, giving a 0-to-14-hour variability in actual age. Pups whose weight at birth was below 5.5 g (i.e. under the 10th percentile) were considered growth retarded and were excluded. Pup's sex was then determined and litter size was adjusted to 10 pups per dam (5 males + 5 females, as possible) to assure a certain equality in feed intake and growth rate. Pups were identified by fur or tail marking using a non-toxic black Permanent marker pen (NN50, Pentel pen). Litters were then assigned to control (CTRL) or sensorimotor restriction (SMR) group. Body weight of pups was assessed daily from P2 to P28 between 8 and 9 AM, i.e. just after cast removal for the SMR group (using a Kern S72 precision balance,  $d = 0.1$  g). We used a total of 40 CTRL and 70 SMR rats (58 males and 52 females), obtained from respectively 4 and 7 litters. Although it is clear that there is a high degree of correlation within the litter (all litter members being subject to the same genetic, nutritional and maternal variables), each subject was treated as an individual (Fox, 1965). In another series of rats (20 CTRL and 20 SMR), pups were euthanized at P8, P15, P21 and P28 and soleus muscle was removed and weighted.

Animal manipulation were performed according to the recommendation of the European Communities Council Directive 2010/63/UE and received agreement of the Regional Committee on the Ethics of Animal

Experiments of Haut de France region (CEEA 75, reference number: APAFIS#2021-020818231865). All efforts were made to minimize the number of animals and their suffering.

### 5.2. Hind limb immobilization

In the SMR group, pups were subjected to transient hind limb immobilization from P1 to P28 for 16 h/day during the dark phase of day, according to Delcour's model (2018a). Briefly, hind limbs were immobilized in extended position with a cast whose size was adapted to the pup growth. These casts did not prevent pups from urinating, defecating or receiving maternal care. Animals were restricted in their movements from 16 PM to 8 AM. The casts were then removed from 8 AM to 16 PM so that pups could move freely for 8 h/day. Protocols were optimized in order to minimize mother/pups separation that did not exceed 15 min per day. CTRL rats were also manipulated in the same way.

### 5.3. Developmental tests

From P1 to P28, sensorimotor development was observed in order to determine the age of onset of the main stages of neonatal development. Sensorimotor tests were performed between 9:00 and 11:00 (righting, posture, negative geotaxis, cliff avoidance) and between 16:00 and 17:00 (eyelid reflex, grasping, placing) in the housing room or next to it. To avoid hypothermia, rat pups were tested under a warm lamp. Tests were performed daily until the reflex was acquired. A reflex was considered acquired if the animal obtained the maximum score on two consecutive days. The following description of tests is adapted from Fox (1965) and Lubics et al. (2005).

**Eye opening.** Eyelids were observed daily from P10. The score was 0 if no eyelid was opened, 1 if one eyelid was opened and 2 if both eyelids were opened (Fig. 2A).

**Eyelid reflex.** The eyelid was gently touched with the rounded end of a metallic flexible rod. The score was 0 if there was no eyelid contraction, 1 if there was the contraction of one eyelid and 2 if the two eyelids contracted.

**Posture.** The animal was placed on a non-slippery surface and the posture was observed. The score was 0 if the pup did not move. An immature posture (score 1) was reflected by dragging of the abdomen when moving, and perpendicular pointing of both fore paws and hind paws relative to the body (Fig. 3A). A mature posture (score 2) was acquired when the pup can lift the abdomen from the surface and both fore paws and hind paws were pointed straight, or parallel to the body, when moving.

**Hind limb/fore limb grasping.** The palm (fore limb) or sole (hind limb) were touched with a thin rod (with a slight pressure). Successful grasping of the rod appeared as flexion of the digits around the rod (Fig. 4A). The score was 0 if there was no grasping, 1 if the grasping was successful by one paw only and 2 if the grasping was successful by both paws.

**Hind limb placing.** The back of the foot was touched with the edge of a flat surface while the animal was suspended by the torso (Fig. 5A). A correct placing reflex was achieved when the rat first withdrew the hind limb and then placed it down on that surface. The score was 0 if the reflex was not successful, 1 if the reflex was successful for 1 leg and 2 if the reflex was successful by both legs.

**Righting.** The pup was held in a supine position with all four paws upright. Righting was achieved (score 1) when the pup was able to roll over to recover a prone position (Fig. 6A). A maximum of 15 s was given to each pup to achieve this goal.

**Cliff avoidance.** The pup was placed at the edge of a flat surface with the fore paws and snout were over the edge (Fig. 7A). The experimenter's hand was placed under the cliff to catch the pup if it fell. The score was 0 if the pup did not move or fell, 1 if it tried to move away from the cliff and 2 if it completely turned away from the edge of the cliff in a

maximum of 30 s.

**Negative geotaxis.** The rat pup was placed head down on a 25°-inclined platform covered with a fine wire grid (4 mm mesh size). The score was 0 if the pup did not move, 1 if the pup turned and 2 if it turned and climbed up and reached the upper rim (Fig. 8A). A maximum of 30 s was given to each pup to achieve this goal.

### 5.4. Data analysis

Statistical analyses were performed using Prism Version 8.4.3 (GraphPad Software, San Diego, US) and the IBM SPSS version 27.0 (IBM Corp, Armonk, New York, US). Quantitative data are presented as mean  $\pm$  SEM. Data normality was determined with the Shapiro-Wilk test. Body weight (BW), muscle wet weight (MWW) and MWW/BW were compared using linear mixed models (fixed effects: group, sex, day; group  $\times$  sex interaction). The residuals were analyzed, with the normality assumption always being met except for BW. Thus, this parameter was investigated with a multiple linear regression model. Reflex ontogeny was analyzed by comparing the mean score of CTRL and SMR groups. To investigate the effects of group, sex, and day, and the interaction between group and sex, on the score for developmental reflexes, the data were submitted to a multiple linear regression model (with scores as dependent variable and day as covariate). Multiple t-tests with correction for multiple comparisons using the Holm-Sidak method were then conducted to determine differences between groups for each day. We also conducted t-tests for comparing time to achieve the test (righting, cliff avoidance and negative geotaxis). Values of  $p < 0.05$  were considered statistically significant, and  $p < 0.1$  were reported as a tendency.

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### CRedit authorship contribution statement

**Orlane Dupuis:** Conceptualization, Formal analysis, Investigation, Writing – original draft. **Melanie Van Gaever:** Investigation. **Valerie Montel:** Investigation. **Julie Dereumetz:** Investigation. **Jacques-Olivier Coq:** Conceptualization, Resources, Writing – review & editing. **Marie-Helene Canu:** Conceptualization, Formal analysis, Investigation, Project administration, Resources, Supervision, Visualization, Writing – original draft. **Erwan Dupont:** Investigation, Methodology, Supervision, Validation, Writing – review & editing.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Data availability

Data will be made available on request.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.brainres.2024.148773>.

